

AD _____

Award Number DAMD17-95-1-5012

TITLE: Cell-Cell Adhesion and Breast Cancer

PRINCIPAL INVESTIGATOR: Stephen W. Byers, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: January 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 4

19991207 054

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE January 1999		3. REPORT TYPE AND DATES COVERED Annual (15 Dec 97 - 14 Dec 98)	
4. TITLE AND SUBTITLE Cell-Cell Adhesion and Breast Cancer				5. FUNDING NUMBERS DAMD17-95-1-5012	
6. AUTHOR(S) Stephen W. Byers, Ph.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20057				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The objective of this proposal is to elucidate the role of cell-cell adhesion and calcium dependent cell adhesion molecules cadherins in breast tumor progression. We will test the hypothesis that in addition to the occasional loss of E-cadherin expression, breast tumor progression is more realistically modeled by a loss of strong cell-cell adhesion resulting from defects in any one or more of the steps (molecules) required for E-cadherin function. During the past year we have published findings showing that invasive E-cadherin negative breast cancer cells express the mesenchymal cadherin, cadherin 11 which may well contribute to the invasive phenotype. Retinoid treatment was found to directly regulate β -catenin/LEF signaling in a manner independent of cadherin function. We showed for the first time that β -catenin regulates contact inhibition, anchorage independent growth, anoikis and radiation-induced growth arrest. The tumor suppressor gene APC was shown to regulate β -catenin ubiquitination and degradation.					
14. SUBJECT TERMS Breast Cancer				15. NUMBER OF PAGES 160	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited		

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

SPJ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

SPJ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

SPJ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

SPJ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

SW. Rye
PI - Signature

1/4/98
Date

TABLE OF CONTENTS:

Front Cover	1
SF298 Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Nature of Problem	5
Background and Previous Work	5
Rationale and Hypothesis to be Tested	7
Methods and Results	7
Conclusions and Recommended Changes	11
Bibliography	12
Appendix	

5. Introduction

Nature of the Problem

Defects in cell-cell adhesion are commonly associated with tumor progression. There is evidence that alterations in the expression of the calcium-dependent cell adhesion molecule E-cadherin occur in a subset of invasive breast cancers and breast cancer cell lines. However, many invasive breast cancers and metastases are E-cadherin positive. Preliminary results indicate that breast tumor progression may more often be accompanied by alterations in the expression and function of several cadherin-associated molecules that are essential for cadherin-mediated cell-cell adhesion. It is the aim of this proposal to test the hypothesis that, in addition to the occasional loss of E-cadherin expression, breast tumor progression is more realistically modeled by a defect in cell-cell adhesion resulting from an alteration in any one or more of the steps (molecules) required for E-cadherin function. We will take two fundamental approaches. Firstly, we will use two methods for "non-specifically" assessing E-cadherin function and cell-cell adhesive strength in breast tumor samples and cell lines. Secondly, we will specifically investigate the molecular mechanisms that lead to defects in cell-cell adhesion by examining (and manipulating) the expression and phosphorylation state of several E-cadherin associated molecules in breast tumors and cell lines.

Background and Previous Work

Cell Adhesion, Cell junctions and Cancer The concept that alterations in cell to cell adhesion and intercellular communication are involved in tumorigenesis and tumor progression is certainly not new (1). However, it is only recently that the molecular basis underlying these changes has begun to be addressed. Homotypic cell-cell adhesion molecules (CAMs) and cell-cell junction molecules have now been implicated as tumor and metastasis suppressor genes in several systems (2-8). A gene often deleted in colon carcinoma (DCC) and associated with colon tumor progression is likely to be a homotypic CAM of the immunoglobulin superfamily (9). Of most interest to the present proposal are the calcium-dependent class of CAMs, cadherins, characteristically expressed by cells of epithelial origin (10,11). Cadherin-mediated adhesion is fundamentally involved in the organization of epithelial tissues during development, and manipulations of cadherin function result in profound disturbances of tissue organization (10-13). Several drosophila tumor suppressor genes are either cadherins or junction associated molecules and reduction in cadherin expression has been associated with the malignant phenotype in many advanced human carcinomas (2-8). In order for cadherins to function in cell-cell adhesion and promote the formation of junctions several other associated molecules need to be expressed (11,14-16). These molecules known as catenins, link the cadherins to the underlying actin cytoskeleton and are probably involved in propagating adhesion-related signalling (16,17). Our preliminary results show that the expression of certain catenins is lost in malignant breast carcinoma cells (16). Other studies have indicated that the phosphorylation state of catenins can also influence the transformed phenotype (17). The mechanism whereby alterations in cadherin-mediated adhesion affects cell proliferation, morphological differentiation and invasion is unknown. All differentiated epithelial cell collectives are linked by gap junctions permeable to intracellular calcium. In this situation, changes in intracellular calcium can be propagated rapidly among communicating cells. It is therefore not surprising that many tumor cells including breast tumor cells are deficient in gap junctional communication ((18) and references therein). Interestingly, transfection of E-cadherin into squamous cell carcinoma cells lacking gap junction

function results in the expression of the gap junction proteins connexins and the assembly of functional gap junctions (19).

E-cadherin in Breast Cancer The presence of lymph node and distant metastases predict poor prognosis for cancer patients. For example, in a large clinical trial, percent treatment failure at 5 years for patients with no lymph node involvement upon histological examination was 13%, for those with 1-3 positive nodes the failure rate was 39% and for those with >4 positive nodes the failure rate was 69% (reviewed in (20)). These results underscore the necessity for research designed to understand the process of metastasis and to discover molecular markers that will predict whether a given tumor is likely to metastasize. The study of E-cadherin and associated protein expression and function in breast cancer cells can potentially provide information pertinent to both of these aims.

Several studies have examined the expression of E-cadherin in human breast cancer tissues (21-23). Loss or reduction of E-cadherin immunostaining was observed in a proportion of samples in each study. Normal breast epithelial structures consistently stain at cell-cell borders for E-cadherin. In one study (22), 53% of 120 tumors had reduced E-cadherin expression (defined as >10% of cells being E-cadherin-negative). The majority of the samples examined in this study were invasive ductal carcinomas, the most common form of breast cancer diagnosed. Loss of E-cadherin expression correlated with poorer differentiation state and with higher stage (T, N and M). In particular, 86% of samples from patients with distant metastasis (M₁) had reduced E-cadherin staining whereas 47% of samples from patients with no known distant metastasis (M₀) had reduced E-cadherin expression (22). Similar results were reported in a smaller study by Gamallo et al. (21). A third study examined a larger number of invasive lobular carcinomas (23). Complete loss of E-cadherin expression was detected in 29 of 35 samples. The remaining 6 samples had a diffuse staining pattern for E-cadherin. The ductal carcinoma samples had variable intensities of staining for E-cadherin although the proportions of E-cadherin-negative cells in each sample was not quantitated. Taken together, these results indicate that E-cadherin expression is lost in a significant proportion of lobular carcinoma specimens and reduced E-cadherin expression correlated with higher stage (poorer prognosis) and poorer differentiation in invasive ductal carcinomas.

One difficulty in comparing the results of E-cadherin staining of tumor tissues is the inconsistency between observers regarding when to classify a tumor as "E-cadherin-positive" or "E-cadherin-negative". In the study by Oka et al. (22), tumors with >10% of cells displaying no E-cadherin immunostaining were classified as having "reduced expression". However, in many studies no such cutoff was used (e.g. (21)). Many studies also describe "diffuse" or "disorganized" or "reduced" staining patterns for E-cadherin (21,23,24). These descriptions may indicate a defect in connection of E-cadherin to the actin cytoskeleton in these samples which could lead to a loss of adhesive function even in the presence of immunoreactive E-cadherin.

Although a trend between increasing stage and reduced E-cadherin expression was observed in breast cancer (22), the ability to predict metastatic spread based on expression of E-cadherin in a primary breast tumor is uncertain. An analysis of E-cadherin expression in 19 lymph node metastases and in their primary tumors was performed (22). Of the primary tumors, six were E-cadherin-positive, five were E-cadherin-negative and eight had a mixed phenotype. Five of the six lymph node metastases from E-cadherin-positive primary tumors were E-cadherin-positive

and one was mixed. All five of the lymph node metastases from E-cadherin-negative primary tumors were E-cadherin-negative. Among the lymph node metastases from the eight mixed primary tumors, three were E-cadherin-positive, two were E-cadherin-negative and two were mixed. The fact that five of the eight lymph node metastases from mixed primary tumors contained E-cadherin-positive indicates that a selection for E-cadherin-negative cells in the metastatic process does not occur.

Rationale and Hypothesis to be Tested The discussion above together with our preliminary data has led us to conclude that E-cadherin expression alone is a poor predictor of breast tumor invasive potential and metastatic spread. Significant loss of heterozygosity (LOH) of chromosomal locus 16q occurs in several carcinomas including breast (25-27). The human E-cadherin gene is localized to 16q22.1 and it is possible that an apparent reduction in staining intensity in some tumors may be due to this LOH. In breast cancer 16q LOH is correlated with distant metastatic spread. Although loss of functional homotypic cell-cell adhesion and intercellular communication are clearly associated with the transformed malignant epithelial cell phenotype this is not necessarily due to loss of cadherin expression or LOH at the cadherin locus. A defect in any one of the molecules involved in cadherin function, or a change in any of the pathways involved in cadherin responsive intra- and inter-cellular signalling could also result in the same phenotype. Other cell-cell adhesion molecules not discussed here are also likely to be affected. In other words, the two important carcinoma cell adhesion related phenotypes of 1) alterations in contact dependent growth and 2) invasion and metastasis, can be achieved in many different ways. Based on our own preliminary results and those in the literature we have calculated that there are several hundred potential routes whereby functional cell-cell adhesion could be altered during carcinogenesis and result in these phenotypes. Bearing in mind that we are limited by current knowledge this number is likely to be conservative. Not surprisingly, more than a dozen lesions in the cadherin-related adhesion system alone have already been uncovered in various carcinomas and cell lines (see discussion above). Whilst it is clearly of great importance to continue cataloging these molecular changes, indeed we propose to do so in one of our specific aims, it is equally important to develop methods in which functional cell-cell adhesion can be assessed directly. Such methods should uncover any defect in cell-cell adhesive strength no matter what the underlying molecular basis.

6. Body

Experimental Design and Methods

Task 1. To test the hypothesis that cell-cell adhesive strength and E-cadherin triton solubility is correlated with functional E-cadherin-mediated cell-cell adhesion. Years 1-4

The first "non-specific" approach that we will use for assessing tumor cell-cell adhesion strength is based on our recently described laminar flow assay (28). Using this assay developed originally to investigate cell-substratum interactions, we hypothesized that we should be able to distinguish cell-cell adhesive strength among tumor cells which express functional and non-functional E-cadherin. We will extend these studies to many more cell lines and to tumors derived from these lines. We will refine our procedures using tumors derived from cells which we already know express functional and non-functional E-cadherin and which are of known adhesive strength *in vitro*. It is not the goal of this specific aim to use these direct assays of cell-cell adhesive strength as a routine screening procedure for breast cancer. For each sample we will correlate cell-cell

adhesion strength with E-cadherin triton solubility. We will restrict our analyses to tumors in which E-cadherin is present (but perhaps non-functional). In this way we can be certain that the cell aggregates that we analyze are derived from the tumor itself rather than any stromal elements which may contaminate it, since these will be E-cadherin negative and will not exhibit calcium-dependent cell-cell adhesion. We are not so interested in E-cadherin negative tumors since these have an obviously demonstrable lesion in cell-cell adhesion.

Task 1. Methods and Results (refer to figures in previous reports)

Most of the experiments which we proposed to carry out in aim 1 have been completed (see previous report). This work has now been published. The remaining aspects of task 1 involve the application of these assay systems to cells that have been isolated from tumors growing in nude mice. These studies are underway but we do not have results yet.

We will first refine our procedures using tumors derived from cells that we already know express functional and non-functional E-cadherin and which are of known adhesive strength *in vitro*. It is not the goal of this specific aim to use these direct assays of cell-cell adhesive strength as a routine screening procedure for breast cancer. For each sample we will correlate cell-cell adhesion strength with E-cadherin triton solubility (see below). We will restrict our analyses to tumors in which E-cadherin is present (but perhaps non-functional). In this way we can be certain that the cell aggregates that we analyze are derived from the tumor itself rather than any stromal elements which may contaminate it, since these will be E-cadherin negative and will not exhibit calcium-dependent cell-cell adhesion. We are not so interested in E-cadherin negative tumors since these have an obviously demonstrable lesion in cell-cell adhesion.

Nude mice tumors In previous studies we have generated tumors in nude mice from several breast carcinoma cell lines. Some of these have been transfected with E-cadherin. We will continue to do this in the present proposal. 5 million carcinoma cells of varying E-cadherin status will be injected into the right upper mammary fat pad of 4-6 week-old athymic female nude mice (BALB/c-nu/nu) and the injection sites observed once or twice a week for the appearance and size of primary tumors. For estrogen responsive cells, 17-beta estradiol pellets (0.72 mg, 60 day release, Innovative Research of America) will be implanted in the interscapular region. After 3-6 weeks mice are sacrificed and tumor tissue used for the preparation of cells and for frozen sections. Cells from the tumors will be analyzed using the laminar flow assay and cell-cell adhesion strength calculated as we described for the cell lines (see previous report and (28))

Task 2. To measure the expression and phosphorylation state of cadherin-associated proteins in breast tumors and cell lines (Years 1-4). We will examine the expression and phosphorylation state of the cadherin-associated proteins alpha catenin, beta catenin and plakoglobin in breast tumors and cell lines.

Task 2. Methods and Results (appendix 1)

The methods for immunoprecipitation and western blot analysis were described in the previous report and have largely been published. At that time we had shown that serine rather than tyrosine phosphorylation was the prominent post-translational modification of β -catenin in breast cancer cells (see previous report). During the second and third grant periods we extended this work to demonstrate that serine phosphorylation of β -catenin has profound effects on its stability

and cellular localization. We have found that β -catenin in breast cancer cells is targeted for ubiquitination and proteosomal degradation by phosphorylation of a specific serine residue at the N-terminal. This serine lies within a consensus sequence which is present in another protein, I κ B α , which is also targeted for ubiquitination by serine phosphorylation. This work is now published (29). More recent results indicate that the product of the tumor suppressor gene APC normally targets β -catenin for ubiquitination (see results in appendix 1, manuscript submitted). This regulation of β -catenin protein levels by serine phosphorylation may well have implications in breast cancer since β -catenin is a central element in the wnt-1 signaling pathway (30). As emphasized in the original proposal wnt-1 overexpression is known to cause breast cancer in mice (31).

Task 3. Statistical analyses (years 3-4). Results will be correlated with tumor stage, blood vessel count, lymph node status, the expression of prognostic markers and period of metastasis-free survival. We are presently accumulating data on phosphorylation status and detergent solubility of cadherins and catenins in breast tumor tissues. This will continue until year 4 when we should have enough material to carry out a statistical analysis

Task 4. To directly examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength (Years 1-4). We will examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion strength by directly examining the effects of kinase inhibitors and plakoglobin transfection on cell-cell adhesion strength using biophysical methods.

Task 4. Methods and Results

To directly examine the role of phosphorylation and plakoglobin expression on breast cancer cell-cell adhesion. In the previous two reports we presented results showing that in two invasive cell lines β -catenin is constitutively tyrosine phosphorylated. We went on to test the hypothesis that this hyperphosphorylation or a lack of plakoglobin is the cause of the failure of transfected E-cadherin to alter the phenotype of the cells. Although we were successful in generating cell lines which expressed plakoglobin we found that increased plakoglobin expression did not make the cells more adhesive even in the presence of E-cadherin. Similarly, simply inhibiting tyrosine phosphorylation did not make the cells more adhesive. As described below, we now propose that it is the increases expression of a mesenchymal cadherin that renders these cells more invasive.

Expression of the mesenchymal cadherin, cadherin.11 in invasive E-cadherin negative breast cancer cells (Cancer Res. In Press, appendix 2).

Our failure to reverse the poorly adhesive phenotype of invasive breast cancer cells by exogenous expression of E-cadherin or plakoglobin or by inhibition of tyrosine kinase activity prompted us to re-examine the cadherin profile of these cells. In our earlier studies we had generated a model in which invasive breast cancer cells had resulted from an epithelial to mesenchymal transition (EMT (32,33)). In contrast to general opinion, recent data in developing systems has demonstrated that during EMTs the resulting mesenchymal cells do in fact express a cadherin, now characterized as cadherin 11(34). We recently investigated the expression of cadherin 11 in breast cancer cells by (see appendix 3). Cadherin 11 is expressed in all the

invasive breast cancer cells and is never expressed with E-cadherin. In addition, we detected the presence of an alternatively spliced form of cadherin 11. These findings support our argument that breast cancer progression may well be in part due to an EMT. In other experiments we found that N-cadherin was expressed in many breast cancer cell lines but was not restricted to those of a mesenchymal phenotype. These data indicate that it is possible that invasive breast cancer cells express cadherin 11 which is in fact essential for their invasive phenotype. Perhaps specific blockade of cadherin 11 could reverse invasion and tumorigenicity. Another aspect of cadherin 11 is of some interest. Although cad 11 is expressed in embryonic mesenchyme it is not normally expressed in adult mesenchymal cells. However, it is expressed in osteoblasts raising the possibility that cad 11 expression by carcinoma cells could increase the opportunity of these cells to metastasize to bone (35). These data raise the possibility that therapeutic strategies directed at cadherin 11 function or expression should be investigated in the context of preventing invasion and metastasis to bone. It is also possible that cadherin 11 expression could be a useful indicator of tumors that are more likely to give rise to invasive and metastatic tumors. In order for us to pursue these goals it is important that we generate good antibodies to cadherin 11. The antibody we use presently is a gift from ICOS Corporation and is barely adequate for immunocytochemistry. As proposed in the previous report, in the 4th year of the grant we have generated polyclonal and monoclonal antibodies directed against three different regions of cadherin 11. The first encompasses the extracellular domain and should produce function-blocking antibodies, the second and third constructs correspond to the cytoplasmic domain and should produce antibodies specific for the two variants. In the final year of the grant we will test these antibodies for staining in paraffin-embedded sections of breast cancers. In addition, in collaboration with Adherex Inc. we have made small peptide inhibitors of cadherin-11 function. In the final year of the grant we will test the ability of these reagents to perturb cadherin-11 function in vitro.

Retinoid Experiments During the fourth year we have continued our work related to the effect of retinoids on the molecular and cellular aspects of cell-cell adhesion and have the following additional results.

1. Retinoic acid-induced cadherin. In the previous report we showed that retinoid treatment results in increased expression of a cadherin and its localization to a triton insoluble pool at the cell membrane. The cadherin was detected using a pan-cadherin antibody. We proposed to identify this cadherin, a task that has proved more difficult than we expected. We are still trying to do this but have also now demonstrated using a dominant/negative cadherin that the ability of retinoids to increase cell-cell adhesion requires cadherin function (MS in preparation). In the final year of the grant we will complete this work and test the hypothesis that the effects of retinoids on cell proliferation also require cadherin function.

2. Retinoic acid and β -catenin/LEF signaling (submitted for publication, appendix 3). As we demonstrated previously, retinoid treatment of SKBR3 cells results in a remarkable differentiation of the cells accompanied by a dramatic increase in the level of β -catenin and decreased cell proliferation. In contrast, other studies have shown that elevated β -catenin, through its interaction with the transcription factor LEF can act as an oncogene (36-38). In the previous report we showed that retinoic acid decreased the ability of endogenous or exogenous β -catenin to regulate β -catenin/LEF signaling. This work is now submitted for publication (appendix 3). In general terms these results showed us that treatment of cells with retinoic acid

can both increase the adhesive function of β -catenin as well as decrease its signaling activity. Clearly, we will be looking into the mechanism of action in the following year.

β -catenin and the cell cycle. In the last two reports we presented preliminary results indicating that cytoplasmic β -catenin levels varied during the cell cycle. In the fourth year of the grant we have extended this work to show that β -catenin regulates contact inhibition, anchorage – independent growth and radiation-induced growth arrest. This work is now submitted for publication (appendix 4)

Conclusions and implications of the completed research

The major achievements of the 4th year of work are:

- 1) Demonstration of a role for the tumor suppressor gene APC in the ubiquitination and degradation of β -catenin (appendix 1-submitted). The newly described role of β -catenin in a growth factor signaling pathway and as a dominant oncogene in several cancers has prompted a flurry of research activity. Our previous results show that the signaling pool of β -catenin in the cytoplasm is regulated by ubiquitination and proteosomal degradation and that mutation of a particular serine (S37) inhibits this process. Work carried out in 1998 strongly implicates the product of the tumor suppressor gene, APC, in the regulation of β -catenin (appendix 1).
- 2) Publication of the cadherin-11 experiments in Cancer Research (appendix 2). The implications of this work are significant. Since cadherin-11 is not expressed on normal epithelial cells or on non-invasive tumor cells it could provide a target for therapies directed at interfering with its function. Such experiments will be carried out in the remaining grant period.
- 3) 9-cis retinoic acid regulates β -catenin/LEF signaling (appendix 3-submitted). Our results show that in addition to its epithelial-differentiation properties, retinoic acid can inhibit the signaling activity of cytoplasmic β -catenin/LEF. Preliminary results indicate that the effects of retinoic acid are mediated directly at the level of β -catenin/LEF transactivation. This is very significant because it might point to a new and perhaps general mechanism whereby retinoids affect differentiation and proliferation.
- 4) β -catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced growth arrest (appendix 4 submitted). Our new data show directly for the first time that β -catenin itself has transforming properties. In keeping with a role for cell adhesion in this process we found that β -catenin influenced the process of contact inhibition rather than growth per se as well as allowing cells to grow in soft agar. β -catenin also inhibited cells from undergoing suspension-induced apoptosis (anoikis) and made cells resistance to radiation-induced growth arrest. This work is significant because it demonstrates a molecular basis for the transforming effects of wnt and APC mutation in breast cancer and provides a mechanism for contact inhibition.

Bibliography

1. Loewenstein, W.R. Junctional intercellular communication and the control of cell growth. *Biochim. Biophys. Acta*, 560: 1-65, 1979.
2. Shiozaki, H., Tahara, H., Oka, H., Miyata, M., Kobayashi, K., Tamura, S., Iihara, K., Doki, Y., Hirano, S., Takeichi, M., and et Expression of immunoreactive E-cadherin adhesion molecules in human cancers. *Am. J. Pathol.* 139: 17-23, 1991.
3. Shimoyama, Y., Hirohashi, S., Hirano, S., Noguchi, M., Shimosato, Y., Takeichi, M., and Abe, O. Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer. Res.* 49: 2128-2133, 1989.
4. Schipper, J.H., Frixen, U.H., Behrens, J., Unger, A., Jahnke, K., and Birchmeier, W. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. *Cancer. Res.* 51: 6328-6337, 1991.
5. Mareel, M.M., Behrens, J., Birchmeier, W., De Bruyne, G.K., Vleminckx, K., Hoogewijs, A., Fiers, W.C., and Van Roy, F.M. Down-regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cells inside tumors of nude mice. *Int. J. Cancer.* 47: 922-928, 1991.
6. Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D., and Birchmeier, W. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 113: 173-185, 1991.
7. Mahoney, P.A., Weber, U., Onofrechuk, P., Blessman, H., Bryant, P., and Goodman, C.S. The fat tumor suppressor gene in drosophila encodes a novel member of the cadherin gene superfamily. *Cell*, 67: 853-868, 1991.
8. Sommers, C.L., Gelmann, E.P., and Byers, S.W. Characterization of transfected E-cadherin in human breast cancer cell lines. *Mol. Cell Biol.* 3: 12431992.(Abstract)
9. Fearon, E.R. and Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell*, 61: 759-767, 1990.
10. Takeichi, M. Cadherins: a molecular family important in selective cell-cell adhesion. *Annu. Rev. Biochem.* 59: 237-252, 1990.
11. Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*, 251: 1451-1455, 1991.
12. Hirai, Y., Nose, A., Kobayashi, S., and Takeichi, M. Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. *Development*, 105: 271-277, 1989.
13. Gallin, W.J., Chuong, C.M., Finkel, L.H., and Edelman, G.M. Antibodies to liver cell adhesion molecule perturb inductive interactions and alter feather pattern and structure. *Proc. Natl. Acad. Sci. U. S. A.* 83: 8235-8239, 1986.
14. Herrenknecht, K., Ozawa, M., Eckerskorn, C., Lottspeich, F., Lenter, M., and Kemler, R. The uvomorulin-anchorage protein alpha catenin is a vinculin homologue. *Proc. Natl. Acad. Sci. U. S. A.* 88: 9156-9160, 1991.
15. Nagafuchi, A., Takeichi, M., and Tsukita, S. The 102 kd cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. *Cell*, 65: 849-857, 1991.
16. Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S., and Takeichi, M. Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. *Cell*, 70: 293-301, 1992.
17. Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S., and Takeichi, M. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J Cell Biol.* 118: 703-714, 1992.

18. Lee, S.W., Tomasetto, C., Paul, D., Keyomarsi, K., and Sager, R. Transcriptional downregulation of gap junction proteins blocks junctional communication in human mammary tumor cell lines. *J. Cell Biol.* 118: 1213-1221, 1992.
19. Jongen, W.M., Fitzgerald, D.J., Asamoto, M., Piccoli, C., Slaga, T.J., Gros, D., Takeichi, M., and Yamasaki, H. Regulation of connexin 43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. *J. Cell Biol.* 114: 545-555, 1991.
20. Yeatman, T. and Bland, K. Staging of breast cancer. In: K.I. Bland and E.M. Copeland (eds.), *The breast: Comprehensive management of benign and malignant diseases*, pp. 313-330, W.B. Saunders and Co. 1991.
21. Gamallo, C., Palacios, J., Suarez, A., Pizarro, A., Navarro, P., Quintanilla, M., and Cano, A. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. *Am. J Pathol.* 142: 987-993, 1993.
22. Oka, H., Shiozaki, H., Kobayashi, K., Inoue, M., Tahara, H., Kobayashi, T., Takatsuka, Y., Matsuyoshi, N., Hirano, S., Takeichi, M., and et Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res.* 53: 1696-1701, 1993.
23. Rasbridge, S.A., Gillett, C.E., Sampson, S.A., Walsh, F.S., and Millis, R.R. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. *J Pathol.* 169: 245-250, 1993.
24. Eidelman, S., Damsky, C.H., Wheelock, M.J., and Damjanov, I. Expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in normal human tissues and tumors. *Am. J. Pathol.* 135: 101-110, 1989.
25. Lindblom, A., Rotstein, S., Skoog, L., Nordenskjold, M., and Larsson, C. Deletions on chromosome 16 in primary familial breast carcinomas are associated with development of distant metastases. *Cancer Res.* 53: 3707-3711, 1993.
26. Nishida, N., Fukuda, Y., Kokuryu, H., Sadamoto, T., Isowa, G., Honda, K., Yamaoka, Y., Ikenaga, M., Imura, H., and Ishizaki, K. Accumulation of allelic loss on arms of chromosomes 13q, 16q and 17p in the advanced stages of human hepatocellular carcinoma. *Int. J Cancer*, 51: 862-868, 1992.
27. Carter, B.S., Ewing, C.M., Ward, W.S., Treiger, B.F., Aalders, T.W., Schalken, J.A., Epstein, J.I., and Isaacs, W.B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. *Proc. Natl. Acad. Sci. U. S. A.* 87: 8751-8755, 1990.
28. Byers, S.W., Sommers, C.L., Hoxter, E., Mercurio, A.M., and Tozeren, A. The role of E-cadherin in the response of tumor cell aggregates to lymphatic, venous and arterial flow: Measurement of cell-cell adhesion strength. *J. Cell Sci.* 108: 2053-2064, 1995.
29. Orford, K., Crockett, C., Jensen, J., Weissman, A.M., and Byers, S.W. Serine phosphorylation-regulated ubiquitination and degradation of beta catenin. *J. Biol. Chem.* 272: 24735-24738, 1997.
30. Cowin, P. and Burke, B. Cytoskeleton-membrane interactions. *Curr. Opin. Cell Biol.* 8: 56-65, 1996.
31. Brown, A.M., Wildin, R.S., Prendergast, T.J., and Varmus, H.E. A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. *Cell*, 46: 1001-1009, 1986.
32. Sommers, C., Heckford, S.E., Skerker, J.M., Worland, P., Thompson, E.W., Byers, S.W., and Gelman, E.P. Loss of epithelial markers and acquisition of vimentin expression in adriamycin- and vinblastine resistant breast cancer cell lines. *Cancer Res.* 52: 5190-5197, 1992.

33. Sommers, C.L., Byers, S.W., Thompson, E.W., Torri, J.A., and Gelmann, E.P. Differentiation state and invasiveness of human breast cancer cell lines. *Breast Cancer Res. Treatment*, 31: 325-335, 1994.
34. Simonneau, L., Kitagawa, M., Suzuki, S., and Thiery, J.P. Cadherin 11 expression marks the mesenchymal phenotype. Towards new functions for cadherins? *Cell Adhes. Commun.* 3: 115-130, 1995.
35. Okezaki, M., Takeshita, S., Kawai, S., Kikuno, R., Tsujimura, A., Kudo, A., and Amman, E. Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. *J. Biol. Chem.* 269: 12092-12098, 1994.
36. Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. Constitutive transcriptional activation by a b-catenin-Tcf Complex in APC^{-/-} colon carcinoma. *Science*, 275: 1784-1787, 1997.
37. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. Activation of b-catenin/Tcf signaling in colon cancer by mutations in b-catenin or APC. *Science*, 275: 1787-1790, 1997.
38. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. Stabilization of b-catenin by genetic defects in melanoma cell lines. *Science*, 275: 1790-1792, 1997.

**The Ubiquitin-Proteasome Pathway and Serine Kinase Activity
Modulate APC Mediated Regulation of β -catenin-LEF Signaling***

Vijayasurian Easwaran[¶], Virginia Song[¶], Paul Polakis[#], and Stephen Byers^{*§}

*[¶]Department of Cell Biology, Georgetown University School of Medicine, and the
Lombardi Cancer Research Center, Washington D.C. 20007, and [#]Onyx
Pharmaceuticals, Richmond, California 94806.*

Contacts: [§] To whom correspondence should be addressed:

Dr. Stephen Byers, E415 The Research Building, GUMC, 3970 Reservoir Rd., NW

Washington DC 20007 Telephone.: (202) 687-1813 Fax: (202) 687-7505

E-mail: byerss@gunet.georgetown.edu.

This work was supported by Grant DAMD1794J-4171 to V.E. The costs of publication of
this article were defrayed in part by the payment of page charges.

This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C
section 1734 solely to indicate the fact.

Keywords: APC, β -catenin, LEF, ubiquitin, proteasome, serine kinase

Running Title: APC mediated Regulation of β -catenin-LEF Signaling

1 **SUMMARY**

2 The tumor suppressor function of APC depends in part, on its ability to bind and
3 regulate the multifunctional protein, β -catenin. β -catenin binds the HMG-box
4 transcription factor LEF/TCF, to directly regulate gene transcription. Using LEF reporter
5 assays we find that APC mediated down-regulation of β -catenin-LEF signaling is
6 reversed by proteasomal inhibitors, in a dose-dependent manner. APC down-regulates wt
7 β -catenin but not the non-ubiquitinatable S37A mutant β -catenin- induced β -catenin-LEF
8 signaling. The bisindolmaleimide-type PKC inhibitor GF-109203X, which prevents β -
9 catenin ubiquitination, decreases the ability of APC to down-regulate β -Catenin-LEF
10 signaling. All these effects on LEF signaling are paralleled by changes in β -catenin
11 protein levels. Lithium a GSK-3 β inhibitor, does not alter the ability of APC to down-
12 regulate β -catenin protein and β -catenin-LEF signaling in the colon cancer cells tested.
13 These results point to a role for a serine kinase other than GSK-3 β , β -catenin
14 ubiquitination, and proteasomal degradation in the tumor suppressive actions of APC.

1 INTRODUCTION

2 Mutations in the tumor suppressor Adenomatous polyposis coli (APC) gene are
3 responsible for tumors that arise in both familial adenomatous polyposis (FAP) and
4 sporadic colon cancers (1-7). APC mutations are almost always truncating, giving rise to
5 proteins lacking C-termini (6,8,9). Efforts to understand how these mutations contribute
6 to cancer have focussed on the ability of APC to bind, and subsequently down-regulate
7 the cytoplasmic levels of β -catenin (10-13).

8 β -catenin is a multifunctional protein that participates in cadherin-mediated cell-
9 cell adhesion and in transduction of the Wnt growth factor signal that regulates
10 development (14,15). Activation of the Wnt growth factor signaling cascade results in the
11 inhibition of the serine/threonine kinase GSK-3 β , and in response β -catenin accumulates
12 in the cytoplasm (16-18). At elevated cytoplasmic levels, β -catenin translocates to the
13 nucleus, interacts with the High Mobility Group (HMG)-box transcriptional activator
14 Lymphocyte Enhancer-binding Factor/ T-cell Factor (LEF/TCF), and directly regulates
15 gene expression (19-22). Mutations that stabilize β -catenin protein are likely to be
16 oncogenic although this has not been shown directly (23). β -catenin is normally degraded
17 by the ubiquitin-proteasome pathway, following serine phosphorylation (24,25).

18 APC mediated tumorigenesis might depend, in part, on its ability to regulate β -
19 catenin signaling (26). However, the specific mechanism by which APC regulates β -
20 catenin signaling is unknown. In this report, we show that the ubiquitin-proteasome

- 1 pathway and the activity of a serine kinase other than GSK-3 β modulate APC mediated
- 2 regulation of β -catenin-LEF Signaling.

1 **EXPERIMENTAL PROCEDURES**

2 *Reagents, Antibodies, and Cells*

3 ALLN, ALLM, Lactacystin, and MG-132 were purchased from Calbiochem. GF-
4 109203X was purchased from Boehringer Mannheim. Ro31-8220 was a gift from Dr.
5 Robert Glazer. The monoclonal anti- β -catenin antibody (Clone#14) and the anti-FLAGTM
6 antibody were purchased from Transduction Laboratories and Kodak, respectively.
7 Affinity purified rabbit polyclonal anti-APC2 and anti-APC3 antibodies (12) were
8 generously provided by Dr. Paul Polakis (Onyx Pharmaceuticals, CA). Affinity purified
9 FITC- conjugated goat anti-rabbit and Texas Red- conjugated goat anti-mouse antibodies
10 were purchased from Kirkegaard and Perry Labs. The SW480 and CACO-2 colon cancer
11 cell lines were acquired from the ATCC and maintained in Dulbecco's modified Eagle's
12 medium with 5% fetal bovine serum and 1% penicillin/streptomycin.

14 *Transfections and LEF-Luciferase reporter assays*

15 Cells were seeded in 12 well-plates at 1×10^5 cells per well. The following day,
16 cells were transiently transfected with 1 μ g of APC constructs, 0.4 μ g of the LEF-reporter
17 pTOPFLASH (optimal motif) (31), 0.008 μ g pCMV-Renilla Luciferase (Promega), per
18 well, using Lipofectamine-Plus reagent according to manufacturers instructions (GIBCO-
19 BRL) for 5 hr. In experiments designed to monitor the effect of APC on β -catenin
20 protein, 0.3 μ g FLAG-tagged wt β -catenin (25) was co-transfected with 0.6 μ g empty

1 vector or APC constructs. This approach facilitated analysis of only the transfected cells,
2 using anti-FLAG antibodies.

3 Cells were treated with indicated levels of the inhibitors for 12-24hr. Luciferase
4 activity was monitored using the Dual-Luciferase Assay System (Promega). The
5 experimental LEF-luciferase reporter activity was controlled for transfection efficiency
6 and potential toxicity of treatments using the constitutively expressed Renilla luciferase.
7 The specificity of APC mediated effects on LEF-reporters were confirmed using
8 pFOPFLASH which harbors mutated LEF binding sites (31), and an unrelated AP-1
9 reporter(32).

10

11 *Immunological procedures*

12 Double-Immunofluorescence staining for APC and β -catenin were according to
13 Munemitsu et al. (11,40). In experiments where FLAG-tagged β -catenin was co-
14 transfected with APC, anti-FLAGTM antibodies (Kodak) were used to detect the β -catenin.

15

1 RESULTS

2 *APC mediated down-regulation of β -catenin-LEF signaling is reversed by proteasomal* 3 *inhibitors*

4 In the SW480 colon cancer cell line, that produce only a mutant APC protein
5 containing aa 1-1337 of the complete 2843-aa sequence, overexpression of wt APC or
6 deletion construct APC 25 (aa 1342 – 2075) but not APC 3 (aa 2130 – 2843) (Fig.1a)
7 can effect a post-translational down-regulation of β -catenin (11,26). The mechanism of
8 APC mediated β -catenin regulation is unknown. Recently, β -catenin was shown to be
9 regulated at the level of protein stability, via proteasomal degradation (24,25). Proteins
10 targeted for degradation by the ubiquitin-proteasome system are first tagged with multiple
11 copies of the small protein ubiquitin, by a highly regulated ubiquitination machinery (27).
12 Poly-ubiquitinated proteins are recognized and rapidly degraded by the proteasome, a
13 large multi-subunit proteolytic complex. Proteasomal degradation plays a critical role in
14 the rapid elimination of many important regulatory proteins, e.g. cyclins and
15 transcriptional activators like NF κ B-I κ B (28). Proteins regulated via proteasomal
16 degradation can be specifically studied using the well characterized proteasome-specific
17 peptidyl-aldehyde inhibitors (29,30).

18 We tested the hypothesis that APC effects the down-regulation of β -catenin-LEF
19 signaling by targeting β -catenin for proteasomal degradation. SW480 cells were
20 transiently transfected with various APC deletion constructs (Fig. 1a), treated with
21 proteasomal inhibitors and β -catenin-LEF signaling was assayed using the LEF-reporter

1 pTOPFLASH (31). Fig. 1b shows that the APC mediated down-regulation of β -catenin-
2 LEF signaling is reversed by a panel of proteasomal inhibitors including ALLN (*N*-
3 acetyl-Leu-Leu-norleucinal), Lactacystin- β lactone, and MG-132, but not DMSO
4 (vehicle) or ALLM (*N*-acetyl-Leu-Leu-methional; Calpain inhibitor II) that effectively
5 inhibits Calpain proteases but has a 100-fold lower potency as a proteasomal inhibitor.
6 The specificity of APC mediated effects on LEF-reporters were confirmed using
7 pFOPFLASH which harbors mutated LEF binding sites and an unrelated AP-1 reporter,
8 neither of which was influenced by APC (31,32). The proteasomal inhibitor ALLN
9 reverses the APC mediated down-regulation of β -catenin-LEF signaling in a dose-
10 dependent manner (Fig. 1c). The effects of APC25 can be completely reversed by the
11 proteasomal inhibitor ALLN, and the effects of wt APC restored to 50-60% of control
12 values. However, the full-length wt APC construct, and not the APC25 deletion
13 construct, was used for all immuno-staining experiments since it was more
14 physiologically relevant (incorporating all the functional domains). SW480 cells were
15 transfected with empty vector or wt APC, and were treated with DMSO (vehicle), or the
16 proteasomal inhibitors ALLN or Lactacystin- β lactone. Double-immunofluorescence
17 staining for APC and β -catenin shows that the APC induced reduction in β -catenin
18 protein (Figures 2 a and b) is reversed by proteasomal inhibitors ALLN (Figures 2 c and
19 d) and Lactacystin- β lactone (Figures 2 e and f).

1 *APC down-regulates wt β -catenin- but not the non-ubiquitinatable S37A mutant form of*
2 *β -catenin- induced LEF signaling.*

3 Mutation of a single serine residue (S37A) within the ubiquitination targeting
4 sequence (UTS) prevents β -catenin ubiquitination (25). Serine mutations in the ubiquitin-
5 targeting sequence of β -catenin occur in a number of different cancers (33-39). At least
6 one of these, S37A, is a stabilizing mutation that renders β -catenin resistant to
7 ubiquitination (25). If indeed APC regulates β -catenin-LEF signaling by targeting β -
8 catenin for proteasomal degradation, then it should not be able to down-regulate the non-
9 ubiquitinatable S37A mutant β -catenin protein or the LEF signaling induced by this
10 stable form of β -catenin. To test this hypothesis, vector or FLAG-tagged- wt or S37A
11 mutant β -catenin constructs were co-transfected with vector or wtAPC and the LEF-
12 reporters, into SW480 cells. β -catenin-LEF signaling was monitored by assaying LEF-
13 reporter activity. Overexpression of both wt and S37A mutant forms of β -catenin
14 increased the basal LEF-reporter activity by about 30%, even against the background of
15 high levels of endogenous β -catenin and β -catenin-LEF signaling in the SW480 cells.
16 S37A β -catenin is more stable than wt β -catenin (in cells that actively degrade β -catenin,
17 e.g. SKBR3 cells), but both forms increased LEF-signaling by comparable levels in
18 SW480 cells (which lack the ability to degrade β -catenin). Figure 3 shows that APC
19 down-regulates wt β -catenin- but not the S37A β -catenin- induced LEF-signaling. The
20 ability of APC to down-regulate the co-transfected FLAG-tagged- wt β -catenin and the
21 S37A β -catenin protein levels was examined by double-immunofluorescence staining

1 using anti-APC 2 and 3 antibodies and anti-FLAG antibodies (Kodak) (40). By double-
 2 immunostaining for both the FLAG epitope and APC, we were able to monitor effects of
 3 APC specifically on the co-expressed forms of β -catenin. Figures 4a (anti-APC) and 4b
 4 (anti-FLAG) show that wt APC effectively down-regulates wt β -catenin. Figure 4c (anti-
 5 FLAG) shows that in concurrent transfections with vector alone and FLAG-tagged wt β -
 6 catenin, the anti-FLAG antibody efficiently detects the wt β -catenin. Figures 4D and E
 7 show that APC does not down-regulate the S37A mutant β -catenin protein. These
 8 findings compliment the observations of Munimitsu *et al.*, and Li *et al.*, that APC
 9 associates with but does not down-regulate β -catenin with an N-terminal deletion (41,42).

10

11 *The bisindoylmaleimide-type PKC inhibitor GF-109203X decreases the ability of APC to*
 12 *down-regulate LEF-signaling, in a dose-dependent manner.*

13 PKC activity is required for wnt-1 growth factor signaling to inhibit GSK-3 β
 14 activity (18). TPA induced down-regulation of DAG-dependent PKCs prevents Wnt from
 15 inhibiting GSK-3 β (18). However, our earlier studies demonstrate that neither the PKC
 16 inhibitor Calphostin C nor TPA-induced down-regulation of PKCs stabilize β -catenin
 17 (25). In contrast, the bisindoylmaleimide-type PKC inhibitor GF-109203X causes a
 18 dramatic accumulation of β -catenin in the cytoplasm (25). The bisindoylmaleimides
 19 inhibit both DAG -dependent and -independent PKC isoforms by competing with ATP
 20 for binding to the kinase. Whereas, Calphostin C and long term TPA treatment inhibit
 21 only DAG-dependent PKC activities. The inhibitor profile implicates DAG-independent,

1 atypical PKC activity in regulating β -catenin stability. These kinase(s) may offer a level
2 of regulation distinct from the DAG-dependent PKC isoforms that regulate Wnt-
3 dependent and GSK-3 β mediated β -catenin signaling (25).

4 The bisindoylmaleimide-type PKC inhibitor GF-109203X prevents β -catenin
5 ubiquitination, but does not inhibit GSK-3 β (25). We tested the hypothesis that GF-
6 109203X will inhibit the ability of APC to regulate β -catenin-LEF-signaling. Figure 5
7 shows that the PKC inhibitor GF-109203X decreases the ability of APC to down-regulate
8 LEF-signaling, in a dose-dependent manner, in SW480 cells. The changes in β -catenin-
9 LEF signaling are reflected by changes in β -catenin protein (Figure 6). Similar results
10 were obtained with another bisindoylmaleimide-type PKC inhibitor Ro31-8220 (data not
11 shown).

12
13 *Lithium(Li⁺) does not inhibit the ability of APC to down-regulate β -catenin - LEF*
14 *signaling.*

15 Physiologically effective concentrations of Li⁺ specifically and reversibly inhibit
16 GSK-3 β activity *in vitro* and *in vivo*, and can mimic the effects of Wnt signaling on β -
17 catenin in mammalian cells (43-46). Treatment of breast cancer cell lines with lithium
18 results in the accumulation of the cytoplasmic, signaling pool of β -catenin (25). Axin, the
19 recently described product of the mouse *Fused* locus, forms a complex with GSK-3 β , β -
20 catenin and APC (47). Axin promotes GSK-3 β dependent phosphorylation of β -catenin,
21 and may therefore help target β -catenin for degradation (48). However, overexpression of

1 Axin inhibits β -catenin-LEF signaling in SW480 colon cancer cells in the absence of
2 functional, wt APC. It is not known if APC promotes GSK-3 β - dependent
3 phosphorylation of β -catenin. Rubinfeld *et al.*, have shown that the APC protein is
4 phosphorylated by GSK-3 β *in vitro*, and suggest that this phosphorylation event is linked
5 to β -catenin turnover (49). It has also been suggested that APC and axin may regulate the
6 degradation of β -catenin by different mechanisms (50).

7 We tested the hypothesis that Li⁺ can inhibit the ability of APC to down-regulate
8 β -catenin-LEF signaling. The colon cancer cell line SW480 was transfected with empty
9 vector or wtAPC, and treated with 10, 20 or 40 mM LiCl or NaCl for 24 hr. The
10 treatments were initiated immediately following the 5 hr transfection period, and thus the
11 cells were exposed to LiCl or NaCl throughout the 24 hr assay period to assure GSK-3 β
12 repression. Figure 6 shows that lithium does not alter the ability of wt APC to down-
13 regulate β -catenin protein. Figure 7 shows that lithium does not significantly alter the
14 ability of wt APC to down-regulate LEF-reporter activity. Similar results were obtained
15 with the CACO2 cell line (data not shown). These experiments were repeated in several
16 different formats incorporating variations in the amount of wt APC transfected, duration
17 of treatment with lithium, and timing of treatment-initiation following transfections.
18 Regardless of these variations, lithium does not inhibit the ability of exogenous APC to
19 down-regulate β -catenin - LEF signaling in the colon cancer cells tested. Lithium
20 treatment also leads to activation of AP-1 -luciferase reporter activity in *Xenopus*
21 embryos, consistent with previous observations that GSK-3 β inhibits c-jun activity

1 (46,51). Concurrent AP-1 transactivation assays also confirmed that GSK-3 β was
2 inhibited in SW480 and CACO-2 cells, following treatment with Lithium (data not
3 shown). These results indicate that GSK-3 β activity (the molecular target of lithium
4 action) is not required for the ability of exogenously expressed APC to down-regulate β -
5 catenin. Recent data indicated that the role of GSK-3 β may be to potentiate assembly of
6 the APC/Axin/ β -catenin complex (48). In our experiments, the high level of APC
7 expressed in the transiently transfected cells may well drive complex assembly in the
8 absence of GSK-3 β . Indeed, in SKBR3 cells lithium treatment causes the accumulation of
9 cytoplasmic β -catenin, and increases β -catenin - LEF signaling (\$, Easwaran and Byers,
10 unpublished observations).

DISCUSSION

Our observations suggest that one function of APC is to down-regulate β -catenin - LEF signaling via the ubiquitin-proteasome pathway. *In vitro* reconstitution experiments designed to explore β -catenin ubiquitination suggested the requirement of key components other than GSK3 β and APC (Easwaran and Byers, unpublished observations). During the course of this study there has been an explosion of data describing novel proteins, including axin, conductin, and Slimb/ β -TrCP as regulators of β -catenin stability (47,52-57). In *Drosophila*, loss of function of Slimb results in accumulation of high levels of Armadillo, and the ectopic expression of Wg responsive genes (56). Recently, the receptor component of the I κ B-ubiquitin ligase complex has been identified as a member of the Slimb/ β -TrCP family (58). Considering the increasing number of similarities between the regulation of I κ B and β -catenin (25), it is tempting to speculate that like I κ B β -catenin ubiquitination occurs in a multi-protein complex that includes kinases, ubiquitin conjugating enzymes, and co-factors. Context-dependent potentiation of this complex by GSK-3 β and other serine-kinase(s), may be regulated by DAG -dependent and -independent PKC activity, respectively. The challenge for future studies will be to determine the exact role of APC in this process.

1 REFERENCES

- 2
- 3 1. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H.,
- 4 Joslyn, G., Stevens, J., Spirio, L., and Robertson, M. (1991) *Cell* **66**, 589-600
- 5
- 6 2. Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W.,
- 7 Groden, J., Stevens, J., Spirio, L., and Robertson, M. (1991) *Cell* **66**, 601-613
- 8
- 9 3. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K.,
- 10 Utsunomiya, J., Baba, S., and Hedge, P. (1991) *Science* **253**, 665-669
- 11
- 12 4. Miki, Y., Nishisho, I., Miyoshi, Y., Horii, A., Ando, H., Nakajima, T., Utsunomiya, J.,
- 13 and Nakamura, Y. (1991) *Jpn. J Cancer Res.* **82**, 1003-1007
- 14
- 15 5. Miyoshi, Y., Ando, H., Nagase, H., Nishisho, I., Horii, A., Miki, Y., Mori, T.,
- 16 Utsunomiya, J., Baba, S., and Petersen, G. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**,
- 17 4452-4456
- 18
- 19 6. Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki,
- 20 Y., Mori, T., and Nakamura, Y. (1992) *Hum. Mol. Genet.* **1**, 229-233
- 21
- 22 7. Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K.,
- 23 Muraoka, M., Takahashi, H., Amada, Y., and Fukayama, M. (1994) *Cancer Res.* **54**,
- 24 3011-3020
- 25
- 26 8. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Willson, J. K.,
- 27 Paraskeva, C., Petersen, G. M., Hamilton, S. R., and Vogelstein, B. (1993) *Proc. Natl.*
- 28 *Acad. Sci. U. S. A.* **90**, 2846-2850
- 29
- 30 9. Polakis, P. (1995) *Curr. Opin. Genet. Dev* **5**, 66-71
- 31
- 32 10. Su, L., Vogelstein, B., and Kinzler, K. W. (1993) *Science* **262**, 1734-1737
- 33
- 34 11. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. (1995) *Proc. Natl.*
- 35 *Acad. Sci. U. S. A.* **92**, 3046-3050
- 36
- 37 12. Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R.,
- 38 Munemitsu, S., and Polakis, P. (1993) *Science* **262**, 1731-1734
- 39
- 40 13. Rubinfeld, B., Albert, I., Porfiri, E., Munemitsu, S., and Polakis, P. (1997) *Cancer*
- 41 *Res.* **57**, 4624-4630
- 42

- 1 14. Gumbiner, B. (1997) *Curr. Opin. Cell Biol.* **7**, 634-640
- 2
- 3 15. Cadigan, K. M. and Nusse, R. (1997) *Genes Dev* **11**, 3286-3305
- 4
- 5 16. Peifer, M., Sweeton, D., Casey, M., and Wieschaus, E. (1994) *Development* **120**, 369-
- 6 380
- 7
- 8 17. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996) *Mol. Cell Biol.* **16**,
- 9 2128-2134
- 10
- 11 18. Cook, D., Fry, M. J., Hughes, K., Sumathipala, R., Woodgett, J. R., and Dale, T. C.
- 12 (1996) *EMBO J.* **15**, 4526-4536
- 13
- 14 19. Clevers, H. and van de Wetering, M. (1997) *Trends Genetics* **13**, 485-489
- 15
- 16 20. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W.,
- 17 Vogelstein, B., and Clevers, H. (1997) *Science* **275**, 1784-1787
- 18
- 19 21. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and
- 20 Kinzler, K. W. (1997) *Science* **275**, 1787-1790
- 21
- 22 22. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin,
- 23 P. J., Vogelstein, B., and Kinzler, K. W. (1998) *Science* **281**, 1509-1512
- 24
- 25 23. Bullions, L. C. and Levine, A. J. (1998) *Curr. Opin. Oncol.* **10**, 81-87
- 26
- 27 24. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) *EMBO J.* **16**,
- 28 3797-3804
- 29
- 30 25. Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M., and Byers, S. W. (1997)
- 31 *JBC* **272**, 24735-24738
- 32
- 33 26. Polakis, P. (1997) *Biochim. Biophys. Acta* **1332**, F127-47
- 34
- 35 27. Ciechanover, A. (1994) *Cell* **79**, 13-21
- 36
- 37 28. Hershko, A. and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67**:425-79, 425-479
- 38
- 39 29. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and
- 40 Goldberg, A. L. (1994) *Cell* **78**, 761-771
- 41
- 42 30. Lee, D. H. and Goldberg, A. L. (1998) *Trends Cell Biol* **8**, 397-403
- 43

31. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Louriero, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997) *Cell* **88**, 789-799
32. Yang, L., Kim, H. T., Munoz-Medellin, D., Reddy, P., and Brown, P. H. (1997) *Cancer Res.* **57**, 4652-4661(Abtract)
33. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997) *Science* **275**, 1790-1792
34. Fukuchi, T., Sakamoto, M., Tsuda, H., Maruyama, K., Nozawa, S., and Hirohashi, S. (1998) *Cancer Res.* **58**, 3526-3528
35. Voeller, H. J., Truica, C. I., and Gelmann, E. P. (1998) *Cancer Res.* **58**, 2520-2523
36. Miyoshi, Y., Iwao, K., Nagasawa, Y., Aihara, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., and Nakamura, Y. (1998) *Cancer Res.* **58**, 2524-2527
37. Palacios, J. and Gamallo, C. (1998) *Cancer Res.* **58**, 1344-1347
38. Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M., and Bodmer, W. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 10330-10334
39. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997) *Science* **275**, 1790-1792
40. Munemitsu, S., Souza, B., Muller, O., Albert, I., Rubinfeld, B., and Polakis, P. (1994) *Cancer Res.* **54**, 3676-3681
41. Munemitsu, S., Albert, I., Rubinfeld, B., and Polakis, P. (1996) *Mol. Cell Biol.* **16**, 4088-4094
42. Li, C., Bapat, B., and Alman, B. A. (1998) *Am. J Pathol.* **153**, 709-714
43. Garcia-Rill, E., Skinner, R. D., Jackson, M. B., and Smith, M. M. (1983) *Brain Res. Bulletin.* **10**, 57-62
44. Edwards, D. L. and Grafstein Bernice| (1983) *Brain Res.* **269**, 1-14
45. Klein, P. S. and Melton, D. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 8455-8459
46. Hedgepeth, C. M., Conrad, L. J., Zhang, J., Huang, H. C., Lee, V. M., and Klein, P. S. (1997) *Dev Biol* **185**, 82-91

- 1
- 2 47. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., and
- 3 Kikuchi, A. (1998) *J Biol Chem* **273**, 10823-10826
- 4
- 5 48. Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998)
- 6 *EMBO J* **17**, 1371-1384
- 7
- 8 49. Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996)
- 9 *Science* **272**, 1023-1026
- 10
- 11 50. Yamamoto, H., Kishida, S., Uochi, T., Ikeda, S., Koyama, S., Asashima, M., and
- 12 Kikuchi, A. (1998) *Mol. Cell Biol* **18**, 2867-2875
- 13
- 14 51. Nikolakaki, E., Coffey, P. J., Hemelsoet, R., Woodgett, J. R., and Defize, L. H. (1993)
- 15 *Oncogene* **8**, 833-840
- 16
- 17 52. Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B., and Polakis, P. (1998)
- 18 *Curr. Biol* **8**, 573-581
- 19
- 20 53. Sakanaka, C., Weiss, J. B., and Williams, L. T. (1998) *Proc. Natl. Acad. Sci. U. S. A.*
- 21 **95**, 3020-3023
- 22
- 23 54. Nakamura, T., Hamada, F., Ishidate, T., Anai, K., Kawahara, K., Toyoshima, K., and
- 24 Akiyama, T. (1998) *Genes Cells* **3**, 395-403
- 25
- 26 55. Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl,
- 27 M., Wedlich, D., and Birchmeier, W. (1998) *Science* **280**, 596-599
- 28
- 29 56. Jiang, J. and Struhl, G. (1998) *Nature* **391**, 493-496
- 30
- 31 57. Marikawa, Y. and Elinson, R. P. (1998) *Mech Dev* **77**, 75-80
- 32
- 33 58. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J.
- 34 S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) *Nature* **396**, 590-594
- 35
- 36
- 37
- 38
- 39

1 *Acknowledgements*

2 We thank Patrice Morin, Hans Clevers and Keith Orford for the wt APC
3 expression plasmid, LEF-reporters, and S37A β -catenin construct, respectively. We thank
4 Allan Weissman for comments on the manuscript.

5

FIGURE LEGENDS

Figure 1. A. The structure of wt APC and APC deletion constructs (26).

B. APC mediated down-regulation of β -catenin-LEF signaling is reversed by proteasomal inhibitors. SW480 cells were transiently transfected with various APC constructs, using Lipofectamine-Plus reagent (Promega). 12 hr post-transfection, the cells were treated with proteasomal inhibitors ALLN, Lactacystin- β lactone, and MG-132, or with DMSO (vehicle) and ALLM (Calpain inhibitor II) for 12 hr. β -catenin-LEF signaling was assayed using the LEF-reporters pTOPFLASH (and pFOPFLASH; data not shown) (31). Raw data was normalized for transfection efficiency and potential toxicity of treatments, using pCMV-Renilla luciferase and the Dual Luciferase Assay System (Promega). The experiment was repeated atleast thrice, with each treatment repeated in triplicate. Error bars represent Standard Deviation.

C. APC mediated down-regulation of β -catenin-LEF signaling is reversed by the proteasomal inhibitor ALLN, in a dose-dependent manner. The transfections were performed as described in figure 1b, and were followed by treatment with the various doses of the proteasomal inhibitor ALLN.

Figure 2. APC mediated down-regulation of β -catenin protein is reversed by proteasomal inhibitors. SW480 cells were transfected with vector or wt APC and were treated with 10 μ M ALLN or 5 μ M Lactacystin- β lactone, or DMSO. Double-immunofluorescence staining for APC and β -catenin was performed according to Munemitsu et al. (11,40).

1

2 Figure 3. APC down-regulates wt β -catenin- but not the non-ubiquitinatable S37A mutant
3 β -catenin- induced LEF signaling. SW480 cells were transfected with empty vector or
4 FLAG tagged -wt β -catenin or -S37A β -catenin, and empty vector or wt APC constructs,
5 and LEF-reporters. 24 hr post-transfection LEF-reporter activity was monitored using the
6 Dual-Luciferase Assay System (Promega).

7

8 Figure 4. APC down-regulates wt β -catenin but not the non-ubiquitinatable S37A mutant
9 β -catenin protein. Transfections were performed as described in figure 3. Double-
10 immunofluorescence staining for APC and β -catenin was performed according to
11 Munemitsu et al. (11,40), except that the tranfected FLAG-tagged β -catenin was detected
12 using anti-FLAG antibodies (Kodak).

13

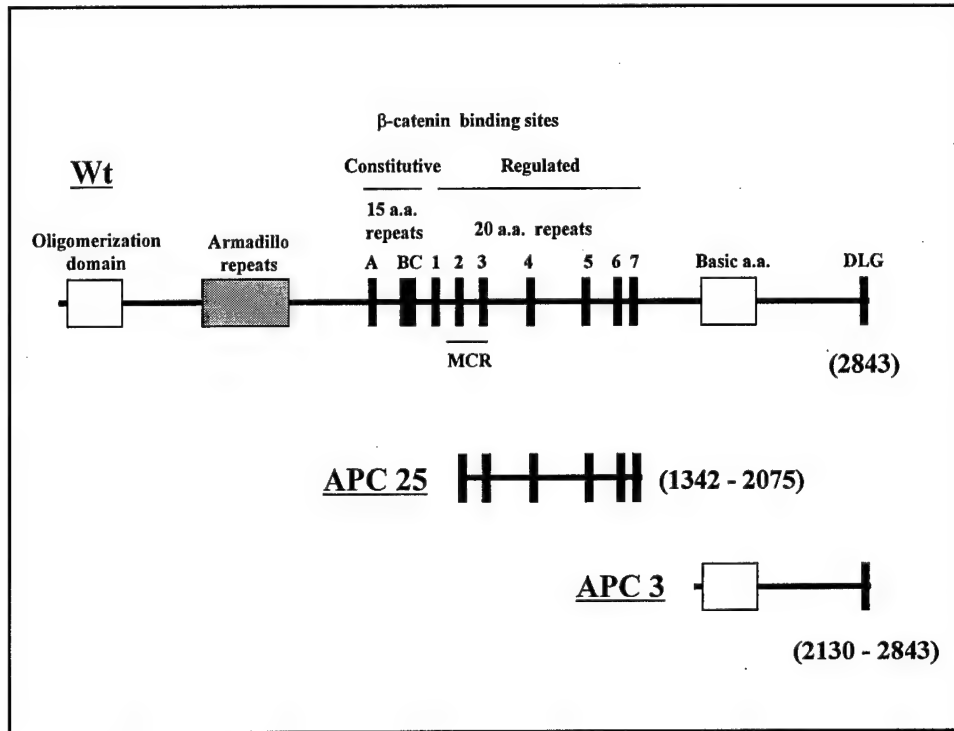
14 Figure 5. The bisindoylmaleimide-type PKC inhibitor GF-109203X, previously shown to
15 inhibit β -catenin ubiquitination, prevents APC from down-regulating β -catenin-LEF
16 signaling, in a dose-dependent manner. SW480 cells were transfected with empty vector
17 or wt APC, and 12 hr post-transfection cells were treated with various concentrations of
18 GF-109203X. 12 hr later LEF-reporter activity was monitored using the Dual Luciferase
19 Assay system.

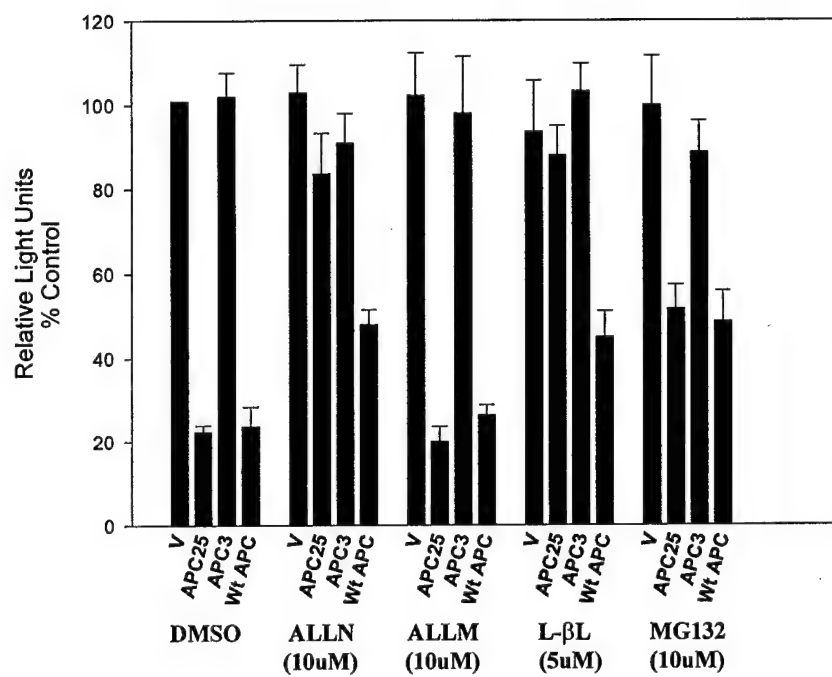
20

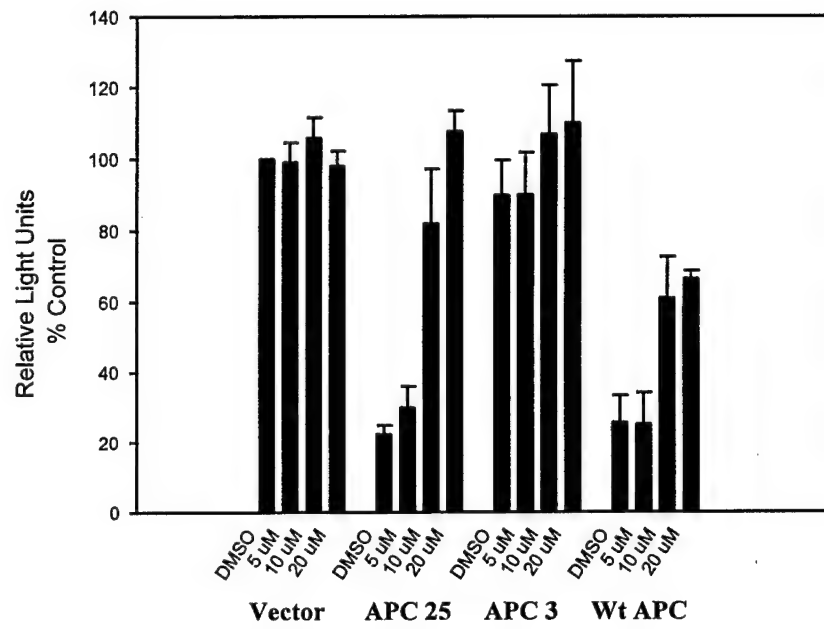
1 Figure 6. The effects of the PKC inhibitor GF-109203X, and Lithium on APC induced
2 reduction in LEF reporter activity are paralleled by changes in β -catenin protein. SW480
3 cells were transfected with empty vector or wt APC and were treated with 5 μ M GF-
4 109203X for 12 hr as described in figure 5. The NaCl or LiCl (20 mM) was added
5 immediately following transfections, and was present through out the 24 hr assay period,
6 to assure GSK-3 β repression. Double-immunofluorescence staining for APC and β -
7 catenin was performed according to Munemitsu *et al.* (11,40).

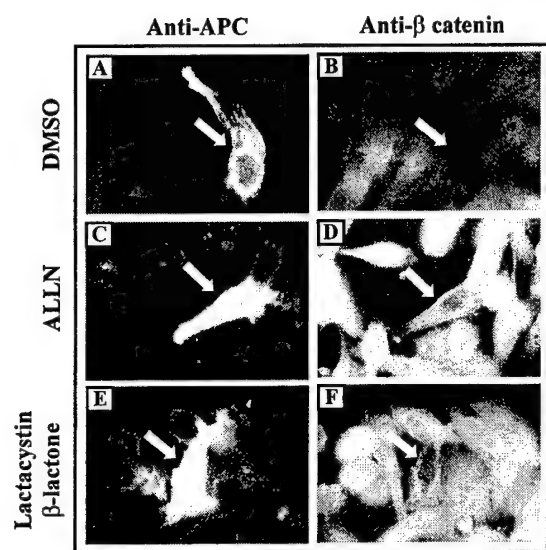
8

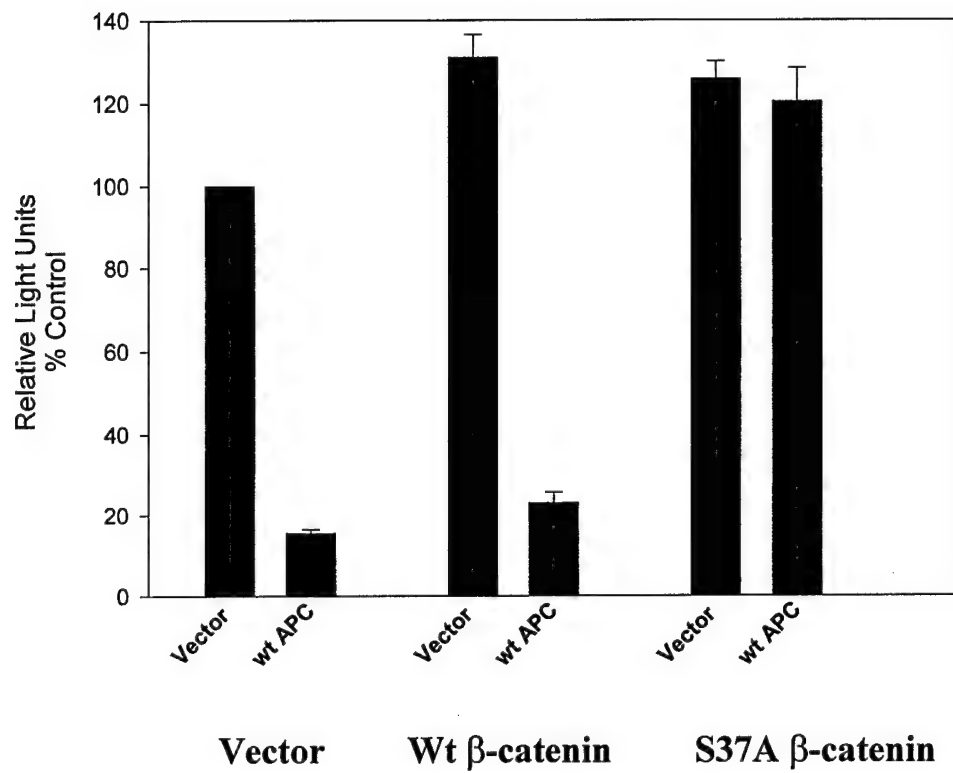
9 Figure 7. Lithium, an inhibitor of GSK3 β also, does not significantly alter the ability of
10 APC to down-regulate LEF-reporter activity. SW480 cells were transfected with empty
11 vector or wt APC. Various concentrations of NaCl or LiCl were added immediately after
12 transfection, to assure GSK-3 β repression. 24 hr later LEF-reporter activity was
13 monitored using the Dual Luciferase Assay system.

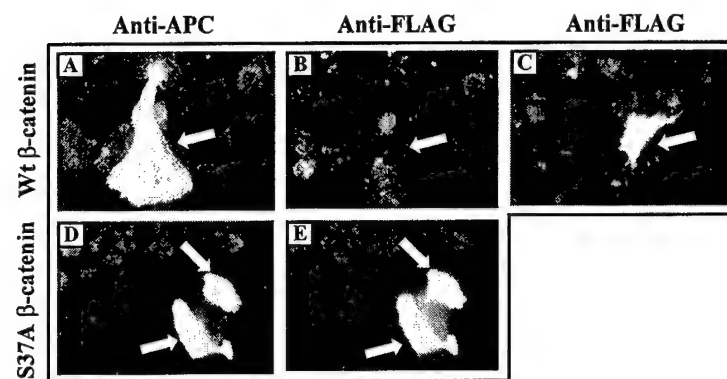


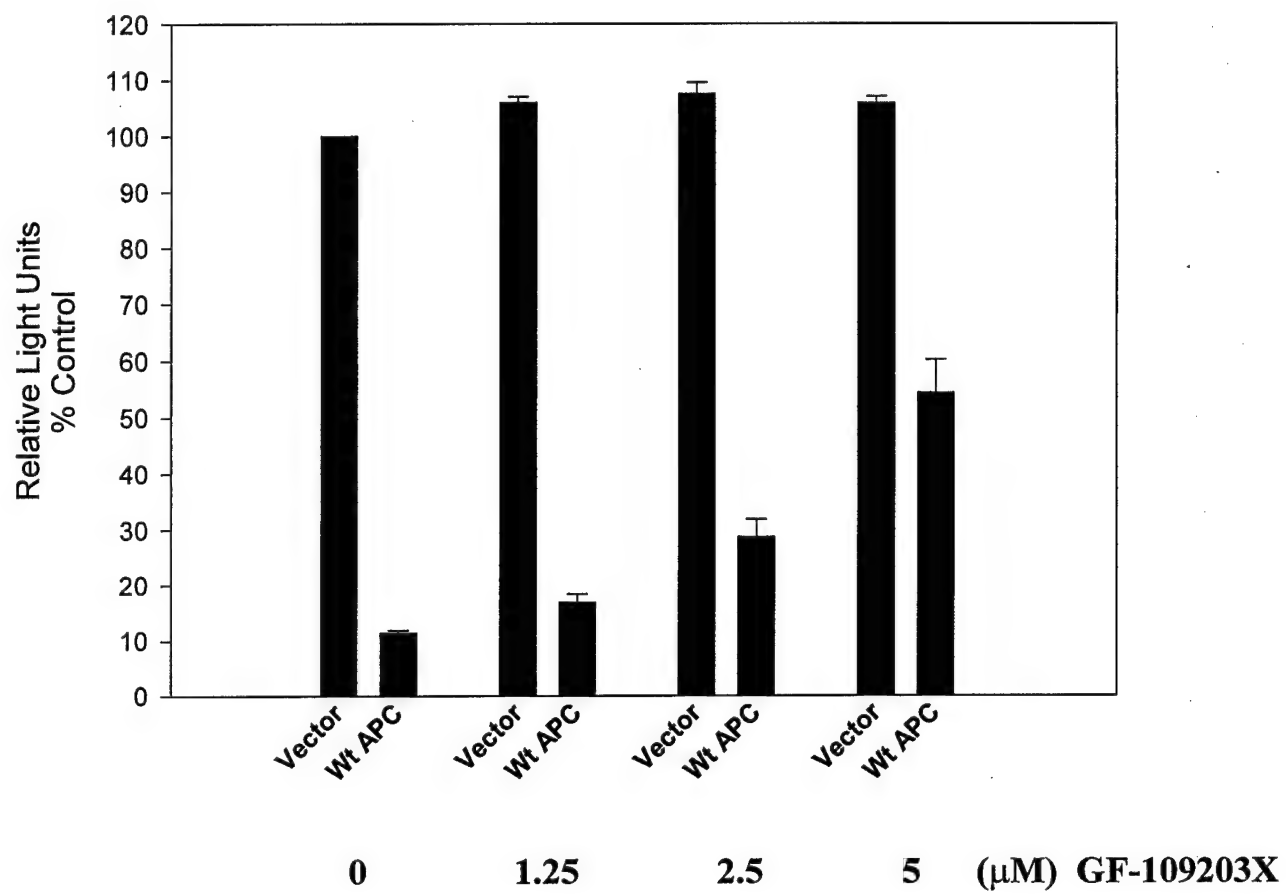


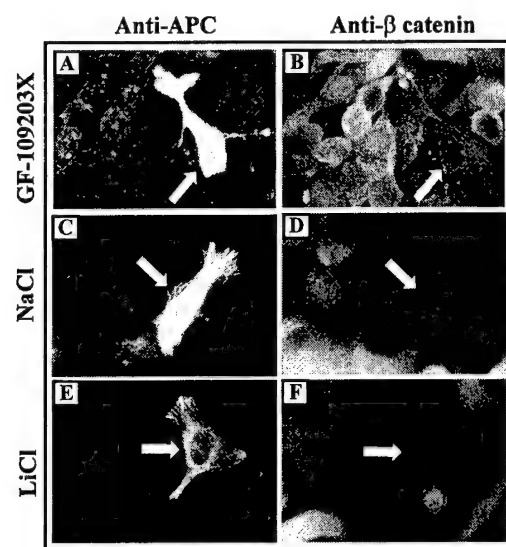


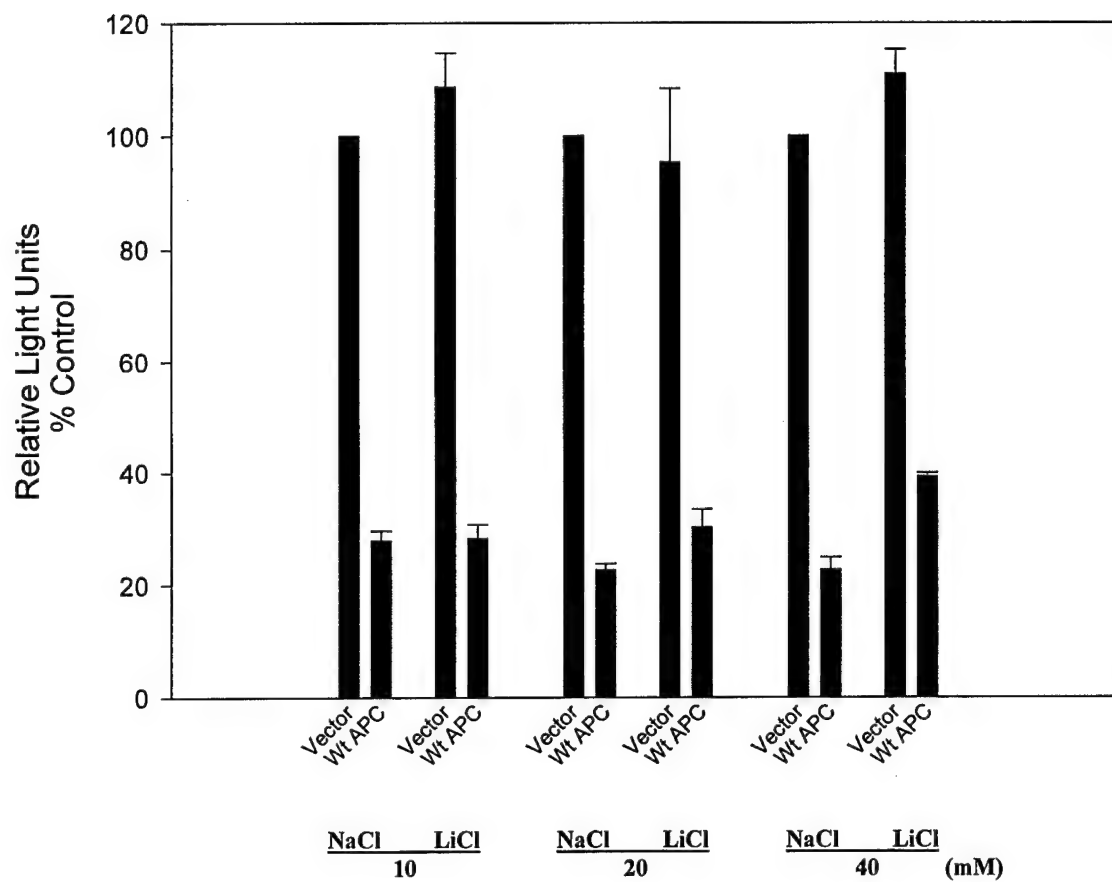












Cadherin-11 is Expressed in Invasive Breast Cancer Cell Lines

**Michael J. Pishvaian[†], Carolyn M. Feltes[†], Patrick Thompson[†], Marion J.
Bussemakers[¶], Jack A. Schalken[¶], and Stephen W. Byers^{†*}**

*[†]The Lombardi Cancer Research Center and the Department of Cell Biology, Georgetown
University School of Medicine, Washington, D.C. 20007; [¶]Urology Research Laboratory,
University Hospital Nijmegen, The Netherlands*

Contacts: *To whom correspondence should be addressed:

Dr. Steve Byers, E412 The Research Building, Georgetown University Medical Center, 3970
Reservoir Road, NW Washington, DC 20007 Phone: (202) 687-1813 Fax: (202) 687-7505

Email: byersS@gunet.georgetown.edu

Running Title: Cadherin-11 in Invasive Breast Cancer Cells

Keywords: Cadherins, Cancer, Invasion, Cadherin-11, Markers

Abstract

In several cancers, including breast cancer, loss of E-cadherin expression is correlated with a loss of the epithelial phenotype and with a gain of invasiveness. Cells that have lost E-cadherin expression are either poorly invasive with a rounded phenotype, or highly invasive, with a mesenchymal phenotype. Most cells lacking E-cadherin still retain weak calcium dependent adhesion, indicating the presence of another cadherin family member. We have now examined the expression of the mesenchymal cadherin, cadherin-11 in breast cancer cell lines. Cadherin-11 mRNA and protein, as well as a variant form are expressed in the most invasive cell lines, but not in any of the non-invasive cell lines. Cadherin-11 is localized to a detergent soluble pool, and is associated with both α - and β -catenin. Immunocytochemistry shows that cadherin-11 is localized to the cell membrane at sites of cell-cell contact as well as at lamellapodia-like projections which do not interact with other cells. These results suggest that cadherin-11 expression may be well correlated with the invasive phenotype in cancer cells, and may serve as a molecular marker for the more aggressive, invasive subset of tumors. Cadherin-11 may mediate the interaction between malignant tumor cells and other cell types that normally express cadherin-11, such as stromal cells or osteoblasts or perhaps even with the surrounding extracellular matrix, thus facilitating tumor cell invasion and metastasis.

1 **Introduction**

2 Cadherins are transmembrane adhesion molecules that mediate calcium dependent cell-
3 cell adhesion. The cadherins are members of a superfamily of related proteins, whose members
4 include the "classical" cadherins, desmosomal cadherins, protocadherins, and products of tumor
5 suppresser genes like c-ret and Fat (1). Members of the classical cadherin subgroup have been
6 shown to be essential for strong cell-cell adhesion and maintenance of tissue integrity and cell
7 polarity (2). They also facilitate, via homophillic adhesion, the differential sorting of cells during
8 morphogenesis (3). Cadherin function is mediated by its connections with the cytoplasmic
9 catenins, α , β , γ (4,5). The catenins link cadherins to the actin cytoskeleton, and have other
10 signaling functions as well (6).

11 The original cadherins (E-,P-, and N-cadherin) were defined based upon their expression
12 patterns in the adult (7). More recently, many other cadherins have been identified, and the
13 classical cadherin subgroup has been subdivided into 2 groups (8-10): the type I cadherins,
14 which include E-,P-, and N-cadherin; and the type II cadherins, which include cadherins-5, -6, -7,
15 -8, -9, -10, -11, and -12. Type I cadherins share common structural features but low amino acid
16 homology with the type II cadherins (9,10). The function and expression patterns of the type I
17 cadherins have been studied extensively but little is known to date about the type II cadherins
18 (3).

19 Previously it was shown in a panel of breast cancer cell lines that E-cadherin expression
20 is lost as cells become more invasive and less differentiated (11,12). Loss of E-cadherin is also
21 associated with the less differentiated, more invasive subset of breast tumors (1). However,
22 exogenous expression of E-cadherin in invasive breast cancer cells did not inhibit their invasive

1 phenotype (11). In addition, blockade of E-cadherin in non-invasive cells prevented cell-cell
2 adhesion, but did not result in an invasive phenotype (12). Instead, E-cadherin expression and
3 the state of differentiation of these cells is representative of their place along a putative epithelial-
4 mesenchymal transition (11,12). As the cells become more fibroblastoid, they acquire the
5 molecular characteristics of fibroblasts, including an increase in vimentin expression, and loss of
6 known junctional proteins including E-cadherin, desmoplakin, and the tight junctional protein,
7 ZO-1 (See Table I). Nevertheless, even the most invasive cell lines exhibited calcium-dependent
8 cell-cell adhesion, suggesting the presence of another functional cadherin (12). Recent work has
9 demonstrated the presence of other cadherins in mesenchymal and fibroblast cells (13,14). One
10 such mesenchymal cadherin is cadherin-11 (15). We now show that cadherin-11 mRNA and
11 protein and a cadherin-11 variant mRNA are expressed in invasive and poorly differentiated
12 breast cancer cell lines. Cadherin-11 is localized to the cell membrane in a detergent soluble
13 complex, where it associates with α -, and β -catenin, and may facilitate tumor cell invasion and
14 metastasis.

1 **Materials and Methods**

2 **Cell Lines**

3 All cell lines were obtained from ATCC and grown in DMEM (Gibco) plus 5% fetal
4 bovine serum (FBS) as described previously (11). A summary of the characteristics of the breast
5 cancer cell lines has been published (11). An expanded table is included below (See table 1).
6 Please note in particular the level of invasiveness of each cell line, as well as the expression of
7 the various adherens junction molecules. MCF-7_{ADR} cells are a variant of MCF-7 cells that are
8 resistant to adriamycin.

10 **RT-PCR**

11 RT-PCR was performed using 0.2 µg (β-actin) or 1.0 µg (Cadherin-11) of total RNA,
12 isolated using the guanidinium isothiocyanate method (16). The following primers were used: β-
13 actin upstream (5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'); β-actin downstream
14 (5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'); cadherin-11 wild type upstream (5'-
15 ACCAGATGTCTGTGTCAGA-3'); cadherin-11 wild type downstream (5'-
16 GTCATCCTTGTCATCTGCA-3'); cadherin-11 variant upstream (5'-
17 CGCCGCGGATCCTTAATGGAACCCCCCTCTC-3'); cadherin-11 variant downstream (5'-
18 CCGCCGGAATTCTCCGTAAGTGTGGTTGGACTCTC-3'). First strand synthesis with the
19 downstream primer and MMLV-RT (Gibco/BRL) was followed by PCR using Taq polymerase
20 (Gibco) after adding the upstream primer. The following cycling parameters were used:
21 Cadherin-11 wild type, 94°C-30 sec., 55°C-30 sec., 72°C-2 min., 35 cycles; Cadherin-11 variant,
22 94°C-1.5 min., 55°C-2 min., 72°C-3 min., 35 cycles. Both parameters could be used for β-actin.

1 The PCR product was run on a 1% agarose gel. The following fragments were amplified: β -
2 actin, a 661 bp fragment that spans an intron to rule out genomic contamination; cadherin-11
3 wild type, a 742 bp fragment from a region encoding part of the extracellular domain; cadherin-
4 11 variant, a 194 bp fragment that encodes most of the C-terminal 75 aa present only in the
5 variant (17).

6 **Northern Blot**

7 20 μ g of total RNA was separated on a 1% agarose gel and transferred to a nylon
8 membrane (Boehringer Mannheim) (16). A 1.6 kb fragment of the cadherin-11 cDNA was
9 labeled using 32 P labeled dCTP and used to probe the blot (DNA fragment donated by Colin
10 MacCalman, University of British Columbia, Vancouver). The blot was hybridized at 50°C
11 overnight, then washed 3 X in 2% SSC at 55°C and 65°C (last wash). The labeled bands were
12 visualized using a phosphoimager. The nylon was then reprobed for GAPDH as a control.

13 **Western Blot**

14 Cells from confluent 10 cm dishes were isolated and dounce homogenized in a hypotonic
15 solution (10 mM Tris, 0.2 mM MgCl_2 , pH 7.5). The homogenate was centrifuged first for 10
16 min. at 3000 X g to remove nuclei. The supernatant was then centrifuged at 150,000 X g for 1
17 hr. The supernatant of this centrifugation, defined as the cytoplasmic fraction, was added to 4
18 volumes of ethanol and the proteins precipitated overnight. The proteins were then collected by
19 ultracentrifugation and solubilized in sample buffer (2% SDS, 60 mM Tris, pH 6.8, 10%
20 glycerol,). The pellet from the first centrifugation was solubilized in a 1% Nonidet P-40 buffer
21 (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0) for 30 min., and clarified in a
22 microcentrifuge for 15 min. The resulting supernatant is the NP-40 soluble membrane fraction.

1 The pellet is the NP-40 insoluble fraction, representing cytoskeletally associated proteins. Both
2 were solubilized in sample buffer. Protein content in the samples was measured (Bio-Rad).

3 Proteins were separated on an 8% reducing polyacrylamide minigel (Novex), transferred
4 to nitrocellulose (Protran) and blocked overnight in 5% milk. The blot was then probed with a
5 monoclonal antibody to cadherin-11 (cad113H, ICOS corporation). This antibody recognizes the
6 extracellular domain of cadherin-11. A secondary peroxidase labeled antibody (Kirkegard and
7 Perry) was added and the bands were visualized by ECL (Amersham). The blots were then
8 stripped at 50°C for 30 min. (stripping solution: 62.5 mM Tris, pH 7.5, 2% SDS, 1.7% (v/v) β -
9 mercaptoethanol), and sequentially reprobed, first with a pancadherin polyclonal antibody
10 (Sigma), next with a monoclonal antibody to E-cadherin (Transduction Labs), and finally with
11 antibodies against α -catenin (Polyclonal from David Rimm, Yale University) or β -catenin
12 (monoclonal antibody, Transduction Labs).

13 **Immunoprecipitation and Immunocytochemistry**

14 For immunoprecipitation, cells from a confluent 10 cm dish were lysed in a 1% NP-40
15 buffer (see above). The lysate was clarified in a microcentrifuge and the supernatant was
16 precleared with 100 μ g of normal rabbit serum. The lysate was immunoprecipitated at 4°C
17 overnight using a polyclonal antibody against either α - or β -catenin (both donated by David
18 Rimm, Yale University). The bound proteins were isolated with protein-A sepharose beads
19 (Boehringer Mannheim), washed 6 times, and boiled in Laemlli buffer with β -mercaptoethanol
20 and the western blot was performed as described above (25).

21 For immunocytochemistry, cells were grown on 18 mm coverslips, then fixed in 2%
22 paraformaldehyde for 15 min., and blocked in 3% ovalbumin for 1 hr at RT. Coverslips were

- 1 incubated in primary antibody (cadherin-11 monoclonal antibody or a β -catenin polyclonal
- 2 antibody) overnight at 4°C, then washed and incubated with Texas Red-conjugated secondary
- 3 antibody for 1 hr. at RT. Coverslips were washed, mounted using Vectashield fluorescence
- 4 mounting medium (Vector), and visualized on a Zeiss microscope.

Results

Cadherin-11 Wild Type and Variant mRNA Expression in Human Breast Cancer Cell Lines

We first examined the expression of cadherin-11 in breast cancer cell lines by RT-PCR. Two sets of primers were used: the first amplifies a region that encodes the extracellular domain of cadherin-11 and thus would recognize both the wild type and variant cadherin-11; the second set amplifies only a portion of the variant cadherin-11 (17). β -actin primers were used as a control. MRC-5, a human embryonic lung fibroblast cell line known to express cadherin-11 was used as a positive control (18). Cadherin-11 wild type (Fig. 1a) and variant (Fig. 1b) mRNA were expressed in five invasive cell lines, MDA-MB-157 (RT-PCR and Northern not shown), MDA-MB-231, BT549, HS578T, and MCF-7_{ADR}. In MCF-7_{ADR} cells, which are considerably less invasive than the others, the variant is expressed at very low levels. Two invasive cell lines, MDA-MB-436 and MDA-MB-435 did not express cadherin-11 nor did any of the non-invasive cell lines. Although a variant cadherin-11 band was amplified from SK-BR-3 cell mRNA, we do not believe that this result represents true expression of cadherin-11 considering all other results were negative (Wild-Type RT-PCR, Northern and Western blots). The RT-PCR results were confirmed by northern blot using a 1.6 kb fragment of the cadherin-11 cDNA as a probe (Fig. 1c). The probe identified a 4.4 kb band from the same five cell lines. Several larger bands can also be seen, but these have yet to be identified. The variant mRNA contains an insertion of 179 bp, but the northern blot did not allow us to differentiate between wild type and variant transcripts. MDA-MB-157 (not shown) and MCF-7_{ADR} cells express much lower levels of mRNA than MDA-MB-231, BT549 or HS578T cells, despite the presence of similar levels of total RNA and GAPDH expression.

Cadherin-11 Protein Expression

We next wanted to examine cadherin-11 protein expression. 10 μ g of protein from a detergent lysate were run on a polyacrylamide gel, and the subsequent blot was probed with an anti-cadherin-11 monoclonal antibody (ICOS, Inc.)(Fig. 2a). This monoclonal antibody recognizes the extracellular portion of cadherin-11 and should recognize both wild type and variant protein. In western blots, cadherin-11 runs as an approximately 120 kDa band. The cell lines which contained cadherin-11 mRNA also expressed cadherin-11 protein. MDA-MB-157 and MCF-7_{ADR} cells express lower levels of cadherin-11 protein. While the putative variant protein would be approximately 7 kDa smaller than the wild type, a band of this size could not be definitively identified on this western blot (17). Several bands significantly smaller than the full length cadherin-11 are present. One of these could represent the variant form if the the protein migrated faster than expected. Alternatively, these bands could represent degradation products. The western blot was reprobed several times to examine the expression of other adherens junction proteins. Probing with a pancadherin antibody revealed that a number of cell lines express another member of the cadherin family (Fig. 2b). The pancadherin antibody is immunoreactive to several members of the cadherin family (the arrow indicates the location of the cadherin-11 band), but is only weakly reactive to E-cadherin. Based upon the strong immunoreactivity and molecular weight (~140 kDa), we believe the pancadherin antibody is identifying N-cadherin in most cell lines. This is consistent with results demonstrated by Hazan, *et al* (19). Interestingly, an anti-E-cadherin western blot demonstrates that E-cadherin and cadherin-11 are never coexpressed (Fig. 2c). Finally, most cell lines express α -catenin (Fig. 2d) and β -catenin (Fig. 2e).

Cadherin-11 Protein is Expressed Primarily in an NP-40 Soluble Pool

We next wanted to determine the solubility of the cadherin-11 protein complex. Cultured cells were separated into three fractions (see methods): cytoplasmic, NP-40 soluble (membrane-bound, non-cytoskeletally associated), and NP-40 insoluble (cytoskeletally associated). 25 μ g of protein from each fraction were run on a polyacrylamide gel, and the blotted proteins were probed with the cadherin-11 monoclonal antibody. In the cells expressing cadherin-11, cadherin-11 protein is found mostly in the NP-40 soluble pool. Some cadherin-11 could also be found in the NP-40 insoluble pool (Fig. 3a). As expected, no cadherin-11 was found in the cytoplasmic pool. By contrast, significant amounts of another cadherin (probably N-cadherin) were found in both the soluble and insoluble pools (Fig. 3b). Also, β -catenin could be found in all three pools, although less was found in the insoluble pool (Fig. 3c).

Cadherin-11 is Localized to the Cell Membrane

In epithelial cells, cadherins typically function as cell-cell adhesion proteins as part of an adherens junction (20). However, cadherin-11 mRNA has been found only in mesenchymal cells which do not usually form adherens junctions (13,15). Immunocytochemistry was performed on paraformaldehyde fixed cells (Fig. 4). Cadherin-11 is expressed at the cell membrane in BT549 and HS578t cells (Fig. 4a, b), but not in MCF-7 cells (Fig. 4d). Staining of BT549 with normal mouse IgG reveals no non-specific staining (Fig. 4c). In BT549 and HS578t cells, cadherin-11 is localized to sites of cell-cell contact, as is typical of cadherin family members. Surprisingly, cadherin-11 is also found in lamellopodia-like extensions and possibly at regions of contact with the substrate.

α -Catenin and β -Catenin Immunoprecipitations

Previously, Shibata, *et al* were able to isolate cadherin-11 by probing a cDNA library with radiolabeled β -catenin protein, thus establishing their interaction *in vitro* (18). We wanted to determine whether or not cadherin-11 interacts with α -catenin and β -catenin *in vivo* (Fig. 5). Confluent cells were lysed in an NP-40 buffer, and the pre-cleared lysate was immunoprecipitated with polyclonal antibodies to either α -catenin or β -catenin (both kindly donated by David Rimm, Yale University). Western Blots were performed first with the cadherin-11 monoclonal antibody (Fig. 5a), then with a pancadherin polyclonal antibody (Fig. 5b), then with a monoclonal antibody to E-cadherin (Fig. 5c), and finally with antibodies to α -catenin (polyclonal) or β -catenin (monoclonal) (Fig. 5d, e). Immunoprecipitation with non-immune rabbit IgG revealed no immunoreactive bands in any of the western blots (data not shown). The western blots confirmed that the same five cell lines express cadherin-11, and that in all five cases cadherin-11 is associated with both α -catenin and β -catenin (except in MDA-MB-157 cells which lack α -catenin). Pancadherin blotting and E-cadherin blotting revealed that several cell lines express other cadherins that are α -catenin and/or β -catenin-associated. Several invasive cells express N-cadherin while E-cadherin expression is restricted to the most differentiated, non-invasive cells (12,19). A few of these cell lines lack α -catenin or β -catenin (See Table I). One particularly striking example cell line is T47D. In these cells both α -catenin and β -catenin are associated with E-cadherin, but not with each other. Nevertheless, these cells are well differentiated and exhibit strong cell-cell adhesion, suggesting that compensatory mechanisms exist in the function of the adherens junction (12). In addition to confirming the results of the western blots, the α -catenin and β -catenin immunoprecipitations identify other

1 defects in the cadherin/ catenin system in various cell lines, which are summarized in Table I.
2 Finally, some proteins which were undetectable by western blot of cell lysates were present
3 detected immunoprecipitations, presumably as a result of the selective concentration afforded by
4 the immunoprecipitation.

5 Previously we published a table that listed the molecular characteristics of several breast
6 cancer cell lines (12). We have now updated that table to include the information on cadherin-11
7 as well as published results on N-cadherin protein, and our preliminary results on N-cadherin
8 mRNA. Note in particular that cadherin-11 is expressed only in the cell lines with a stellate
9 morphology. These are the most invasive cell lines, and they all lack E-cadherin.

Discussion

Cadherin-11 is Expressed in Invasive, Fibroblastoid Breast Cell Lines

Loss of E-cadherin expression or function is associated with a more invasive, less differentiated phenotype in cancer cell lines, and primary tumor samples (11,12). In earlier studies, we established three subsets from a panel of breast cancer cell lines (11,12). E-cadherin expressing cell lines (which all lack vimentin) were poorly invasive in Boyden Chamber assays and well-differentiated, forming tight cell clusters in Matrigel. Cell lines lacking E-cadherin and vimentin were also poorly invasive, but only moderately differentiated, forming loose cell aggregates in Matrigel. Finally, cell lines lacking E-cadherin, but expressing vimentin were highly invasive and poorly differentiated, and had a stellate, fibroblastoid morphology. However, transfection of E-cadherin into these cells did not reverse the invasive phenotype (12). These results indicated that loss of E-cadherin expression or function may be necessary, but is not sufficient for the establishment of invasive, highly malignant tumors.

Despite the lack of E-cadherin expression, most of the invasive cells exhibited calcium-dependent adhesion, indicating the presence of another member of the cadherin family (11). Recent work has demonstrated the expression of cadherins in fibroblastic and mesenchymal cells. N-cadherin is expressed in several types of mesenchymal tissue as well as in cultured fibroblasts (14). In addition, cadherin-11 expression is restricted to mesenchymal cells in the developing mouse (13,15,21), and is expressed in stromal cells in the adult (18). Hazan, *et al* have shown that N-cadherin expression in breast cancer cell lines is limited to most of the invasive, fibroblastoid cells (19). They proposed that N-cadherin expression is restricted to less differentiated cells, and that the expression of N-cadherin facilitates the interaction of tumor cells with the underlying stroma. Our preliminary results indicate that N-cadherin mRNA (but not a

1 pan-cadherin immunoreactive protein) is also present in several non-invasive cell lines (See
2 Table I, Data not Shown). Consequently, it may be interesting to explore the post-transcriptional
3 regulation of N-cadherin, and its possible relevance in breast cancer.

4 We now present evidence that cadherin-11 is expressed in breast cancer cell lines that
5 lack E-cadherin but express vimentin and are highly invasive and poorly differentiated. RT-
6 PCR, northern blot, and western blot analyses confirm that five of seven invasive breast cancer
7 cell lines express cadherin-11. Cadherin-11 was previously found to be expressed only in
8 embryonic mesenchymal tissues, osteoblasts, and invasive tumors of the stomach and the kidney
9 (17,18,21,22). Furthermore, cadherin-11 has never been shown to be expressed in E-cadherin
10 expressing cell lines or tissues (21,22). This indicates that cadherin-11 is a very specific marker
11 for only the most invasive subset of cancer cell lines. Although we have been unable to
12 determine the level of expression of cadherin-11 in human tumor tissue, the results obtained
13 using cancer cell lines suggests that cadherin-11 is a potential molecular marker, and could be
14 used to identify highly malignant tumors that would require more aggressive therapy. Moreover,
15 our results together with those of Hazan, *et al* show that invasive cells express cadherin-11, N-
16 cadherin, or both, and indicate that detection of these molecules could identify with confidence
17 highly malignant tumors.

18 The five cell lines that express cadherin-11 also express a variant of cadherin-11. This
19 variant arises from a 179 bp insertion that results in a protein that lacks the majority of the wild
20 type cytoplasmic domain, including the catenin-binding regions (17). If the cadherin-11 variant
21 is expressed as a functional protein, it could act as a dominant-negative and reduce cadherin-
22 mediated cell adhesion. In fact, expression of this variant mRNA has been associated with

1 invasive tumors (17,23). However, using cell extracts we were unable to definitively identify a
2 protein product of a size anticipated from the variant mRNA sequence.

3 *Cadherin-11 Associates with α - and β -catenin*

4 Cadherin-11 is a typical type II cadherin, and can mediate calcium dependent cell-cell
5 adhesion. Previously, Shibata, *et al* have shown that cadherin-11 interacts with β -catenin *in vitro*
6 (18). We have shown here that cadherin-11 forms complexes containing both α - and β -catenin
7 *in vivo*. However, in the invasive breast cancer cell, BT549 cadherin-11 is found predominantly
8 in a detergent soluble pool, indicating that it is not associated with the cytoskeleton. In the same
9 cells a pan-cadherin reactive protein (probably N-cadherin) is found in the detergent insoluble
10 pool. Thus, cadherin-11 is not found in a typical adherens junction in which a cadherin is linked,
11 through the catenins, to the actin cytoskeleton. Adherens junctional cadherins are also localized
12 to sites of cell-cell contact. However, our immunocytochemistry shows that cadherin-11 is also
13 found at lamellapodia-like extensions, and possibly to regions of cell-substrate contact. These
14 results suggest that cadherin-11 may have several functions. It may function in a transient form
15 of cell-cell adhesion that involves the cytoplasmic catenins, but is not associated with the
16 cytoskeleton. The cadherin-11 complex may also have a role in contacting the cell-matrix,
17 particularly in leading extensions of the cell. Such a complex would be much more transient and
18 could facilitate the ability of a motile cell to interact with its surroundings, which could include
19 both matrix proteins, as well as other mesenchymal cells. These potential functions of cadherin-
20 11 have implications with respect to the invasive and metastatic ability of tumor cells.

1 *Functional Significance of Cadherin-11*

2 Cadherin-11 could be involved in cell and matrix recognition that may facilitate cell
3 motility and may also be essential for the loose aggregation of cell types that is necessary in
4 tissue morphogenesis. These hypotheses are supported by the following observations: first,
5 cadherin-11 expression is associated with invasive cells, both during normal stages of
6 embryogenesis and in invasive tumor cells (17,18,21,24). This invasion may be facilitated by the
7 association of the invasive cadherin-11 cells with the surrounding mesenchymal cells, which also
8 express cadherin-11, as well as with the surrounding matrix. For example, during embryogenesis
9 mesenchymal cells express cadherin-11 (15). In addition, cells that undergo an epithelial to
10 mesenchymal transition also begin to express cadherin-11 as they invade the surrounding tissue
11 to form new structures (21). This can be seen during branching morphogenesis of lungs and
12 kidneys, and also in the formation of the nasal septum, skin, vibrissae, teeth and various glands
13 (21). Shibata, *et al* show that in a panel of gastric tumors (both primary tumors and tumor cell
14 lines), cadherin-11 is only expressed in signet ring cell carcinomas, which are typically
15 infiltrative (18). They suggest that the expression of cadherin-11 in the tumor cells may allow
16 for interactions with the underlying stroma that would facilitate invasion. Thus, unlike other
17 cadherins, such as E-cadherin and cadherin-6, which have invasion suppressor function,
18 cadherin-11 may actually enhance tumor cell invasiveness, and may be a new target for treatment
19 (11).

20 Next, cadherin-11 may be essential for the loose aggregation of cell types. For example,
21 during embryogenesis, the expression of cadherin-11 is increased dramatically in cells
22 undergoing the epithelial-mesenchymal transition that precedes the formation of various
23 structures, and also in regions of mesenchymal condensation, such as occurs prior to

1 chondrogenesis (13). The association and loose aggregation of cells may depend upon cadherin-
2 11 and could be extremely significant in cancer. For example, it is possible that cadherin-11 may
3 act to specifically target metastatic tumor cells to sites which express cadherin-11.
4 Consequently, expression of cadherin-11 may facilitate association of metastatic cells with
5 cadherin-11 expressing osteoblasts in the bone, thus establishing a bony metastasis.

6 These studies indicate that cadherin-11 expression is associated with invasive breast
7 cancer, and may play a significant role in facilitating tumor cell invasion and the formation of
8 metastatic tumors. Elucidation of the functions and regulation of cadherin-11 may enhance our
9 understanding of the roles of cadherins in invasive cancer and mesenchymal cells, and may
10 provide future targets for therapy.

11
12 **Acknowledgments:** We would like to thank the following: ICOS Corporation for providing the
13 cadherin-11 monoclonal antibody; Dr. David Rimm, Yale University, for providing polyclonal
14 antibodies to α - and β -catenin; and Dr. Colin MacCalman, University of British Columbia,
15 Vancouver for providing the cDNA fragment of cadherin-11 used in the Northern Analysis.

16 This work was funded by a grant from the U.S. Department of Defense to SWB. MJP
17 and CMF are recipients of graduate fellowships from the Department of Defense.

1 Bibliography

- 2 1. Takeichi, M. Cadherins in Cancer: Implications for Invasion and Metastasis. *Curr Opin Cell*
3 *Bio*, 5: 806-811, 1993.
- 4 2. Takeichi, M. Cadherin: A Molecular Family Important in Selective Cell-Cell Adhesion. *Annu*
5 *Rev Biochem*, 59: 237-252, 1990.
- 6 3. Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*, 251:
7 1451-1455, 1991.
- 8 4. Gumbiner, B.M. and McCrea, P.D. Catenins as mediators of the cytoplasmic functions of
9 cadherins. *J Cell Sci, Sup 17*: 155-158, 1993.
- 10 5. Kemler, R. From cadherins to catenins-cytoplasmic protein interactions and regulation of cell
11 adhesion. *Trends Gen. 9*: 317-321, 1993.
- 12 6. Gumbiner, B.M. Signal transduction by β -catenin. *Curr. Opin. Cell Biol.* 7: 634-640, 1995.
- 13 7. Takeichi, M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis.
14 *Development*, 102: 639-655, 1988.
- 15 8. Suzuki, S., Sano, K., and Tanihara, H. Diversity of the cadherin family: Evidence for eight
16 new cadherins in nervous tissue. *Cell Regul.* 2: 261-270, 1991.
- 17 9. Tanihara, H., Sano, K., Heimark, R., John, T.S., and Suzuki, S. Cloning of five new cadherins
18 clarifies characteristic features of cadherins extracellular domain and provides evidence of two
19 structurally different types of cadherin. *Cell Adhesion and Communication*, 2: 15-26, 1994.
- 20 10. Tanihara, H., Kido, M., Obata, S., Heimark, R.L., Davidson, M., St.John, T., and Suzuki, S.
21 Characterization of cadherin-4 and cadherin-5 reveals new aspects of cadherins. *J Cell Sci.* 107:
22 1697-1707, 1994.

- 1 11. Sommers, C.L., Thompson, E.W., Torri, J.A., Kemler, R., Gelmann, E.P., and Byers, S.W.
2 Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: Relationship to
3 morphology and invasive capacities. *Cell Growth Diffn.* 2: 365-372, 1991.
- 4 12. Sommers, C.L., Byers, S.W., Thompson, E.W., Torri, J.A., and Gelmann, E.P.
5 Differentiation state and invasiveness of human breast cancer cell lines. *Breast Cancer Res.*
6 *Treatment*, 31: 325-335, 1994.
- 7 13. Simonneau, L., Kitagawa, M., Suzuki, S., and Thiery, J.P. Cadherin 11 expression marks the
8 mesenchymal phenotype. Towards new functions for cadherins? *Cell Adhes. Commun.* 3: 115-
9 130, 1995.
- 10 14. Matsuyoshi, N. and Imamura, S. Multiple cadherins are expressed in human fibroblasts.
11 *Biochem. Biophys. Res. Commun.* 23: 355-358, 1997.
- 12 15. Hoffmann, I. and Balling, R. Cloning and expression analysis of a novel mesodermally
13 expressed cadherin. *Dev. Bio.* 169: 3461995.
- 14 16. Sambrook, J., Fritsch, E., and Maniatis, T. Molecular cloning: A laboratory manual. Cold
15 Spring Harbor, NY. Cold Spring Harbor Laboratory, 1989.
- 16 17. Okazaki, M., Takeshita, S., Kawai, S., Kikuno, R., Tsujimura, A., Kudo, A., and Amann, E.
17 Molecular cloning and characterization of OB-cadherin, a new member of cadherin family
18 expressed in osteoblasts. *JBC*, 269: 12092-12098, 1994.
- 19 18. Shibata, T., Ochiai, A., Gotoh, M., Machinami, R., and Hirohashi, S. Simultaneous
20 expression of cadherin-11 in signet-ring cell carcinoma and stromal cells of diffuse type gastric
21 cancer. *Cancer Letters*, 99: 147-153, 1996.

- 1 19. Hazan, R.B., Kang, L., Whooley, B.P., and Borgen, P.I. N-cadherin promotes adhesion
2 between invasive breast cancer cells and the stroma. *Cell Adhesion and Communication*, 4: 399-
3 411, 1997.
- 4 20. Boyer, B. and Thiery, J.P. Epithelial cell adhesion mechanisms. *J. Membr. Biol.* 112: 97-108,
5 1989.
- 6 21. Kimura, Y., Matsunami, H., Inone, T., Shimamura, K., Uchida, N., Ueno, T., Miyazaki, T.,
7 and Takeichi, M. Cadherin-11 is expressed in association with mesenchymal morphogenesis in
8 the head, somite, and limb bud of early mouse embryos. *Dev. Biol.* 169: 347-358, 1995.
- 9 22. Shimazui, T., Girolodi, L.A., Bringuier, P.P., Oosterwijk, E., and Schlaken, J.A. Complex
10 cadherin expression in renal cell carcinoma. *Cancer Res.* 56: 3234-3237, 1996.
- 11 23. Kools, P.F.J., Hogendoorn, P.C.W., and Van Roy, F. Expression in mesenchymal tumors of
12 alternative cadherin-11 transcripts encoding truncated adhesion molecules: a mechanism for
13 acquiring invasive properties? *Clin. Exp. Metas.* 14: 52, 1996.
- 14 24. MacCalman, C.D., Furth, E.E., Omigbodun, A., Brunner, M., Contifaris, C., and Strauss,
15 J.F.I. Regulated expression of cadherin-11 in human epithelial cells: A role for cadherin-11 in
16 trophoblast-endometrium interactions. *Dev. Dynamics*, 206: 201-211, 1996.
- 17 25. Laemlli, U.K. Cleavage of structural proteins during assembly of the head of bacteriophage
18 T4. *Nature*, 227: 680-685, 1970.

Cell Line	Mitrigl	Invasion	ZO-1	DP-1/1	E-cad	α -Catenin	β -Catenin	Plak	NcadProt	NcadRNA	Cad11 WT	Cad11 var	Vimentin
BT483	Fused	2	3	3	2	2	2	2	ND	ND	ND	ND	0
MDAMB-175-7	Fused	2	3	2	2	2	2	2	ND	1	0	ND	0
MCF-7	Fused	2	3	2	2	2	2	2	0	0	0	0	0
ZR-75-B	Fused	2	3	2	2	2	2	1	ND	1	0	ND	0
T47D	Fused	2	2	2	2	2	2	2	ND	1	0	0	0
BT474	Fused	2	0	2	2	2	2	2	0	2	0	0	0
MDAMB-361	Fused	2	0	2	2	2	2	1	0	ND	ND	ND	0
MDAMB-468	Fused	2	0	2	1	0	1	1	0	2	0	0	0
SK-BR-3	Spherical	2	2	1	0	2	1	1	0	ND	0	0	0
CAVA-1	Spherical	2	2	2	0	1	1	1	ND	1	0	0	0
MDAMB-453	Spherical	2	0	1	0	2	1	1	0	3	0	0	0
MDAMB-134	Spherical	2	0	1	0	1	1	1	ND	3	0	0	0
MDAMB-436	Stellate	3	2	0	0	2	2	1	2	3	0	0	2
MDAMB-435	Stellate	3	0	0	0	2	4	ND	2	3	0	0	2
MCF-7 _{ADR}	Stellate	3	0	0	0	2	2	1	ND	3	2	2	2
MDAMB-157	Stellate	3	ND	ND	0	0	3	ND	ND	ND	2	2	ND
MDAMB-231	Stellate	4	0	0	0	2	2	1	0	ND	3	2	2
BT549	Stellate	4	0	0	0	2	2	1	2	ND	3	2	2
HS578t	Stellate	4	0	0	0	2	2	0	2	3	3	2	2

Table 1: Molecular Characteristics of Several Breast Cancer Cell Lines This is based on a table published previously by Sommers, *et al* (12). Data on N-cadherin protein is from Hazan, *et al* (19). Unless specified otherwise, the data represent the presence or absence of mRNA and protein, using the following scale: 0=Negative; 1=+/-; 2=+; 3=++; 4=+++; ND=Not Determined

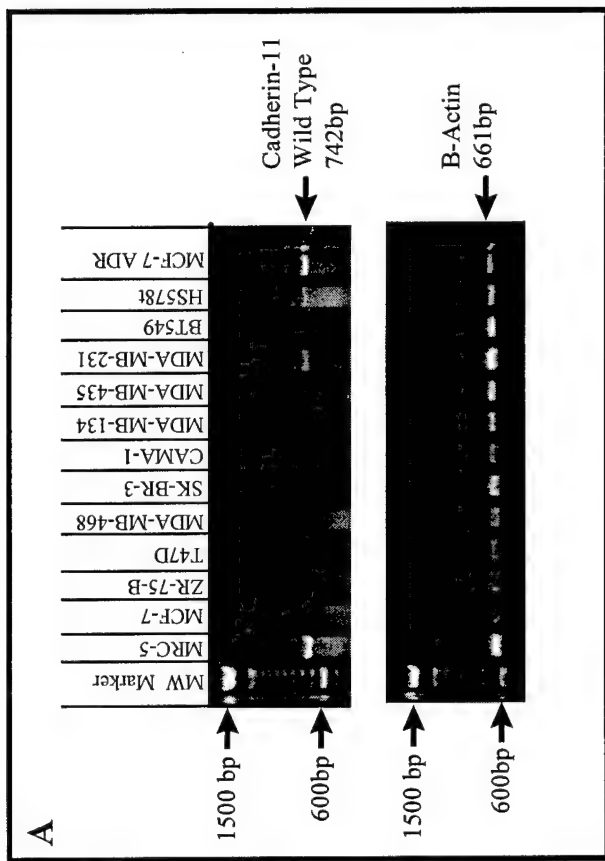
Figure 1. Cadherin-11 mRNA Expression RT-PCR and Northern blot analysis show that cadherin-11 mRNA is expressed in only the most invasive cell lines. A and B) 1 μ g (cadherin-11 wild type and variant) or 0.2 μ g (β -actin) of total RNA was used for the reaction. The primers amplified the following fragments: Cadherin-11 wild type-742 bp (A, upper); Cadherin-11 variant-194 bp (B, upper); β -actin-661 bp (A and B, lower). C) 20 μ g of total RNA was run on an agarose gel and transferred to nylon. The membrane was probed sequentially with a cadherin-11 cDNA fragment (upper), or a GAPDH cDNA probe (middle). The ethidium bromide stained agarose gel shows the 28S and 18S RNA bands clearly (lower).

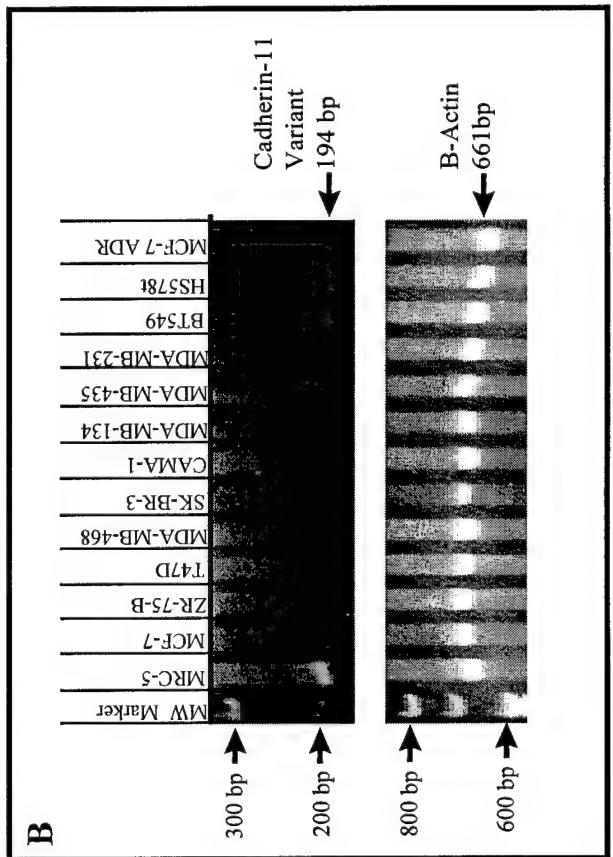
Figure 2. Cadherin-11 Western Blot 10 μ g of protein from an NP-40 soluble pool were run on PAGE. The subsequent nitrocellulose blot was probed with the following antibodies: A) Cadherin-11 monoclonal antibody; B) Pancadherin polyclonal antibody; C) E-cadherin monoclonal antibody; D) β -catenin monoclonal antibody; E) α -catenin polyclonal antibody. The open arrow marks the location of the cadherin-11 band on the pancadherin and E-cadherin blots.

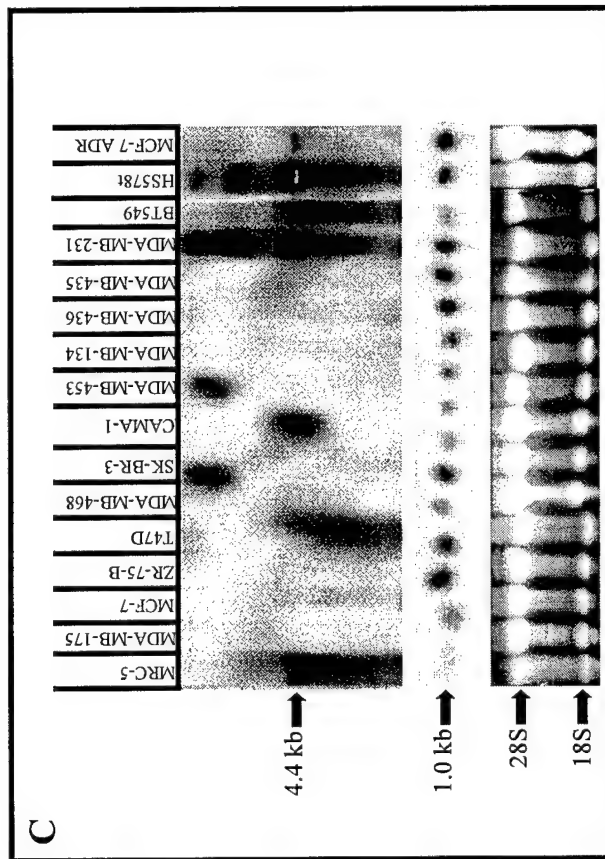
Figure 3. Cadherin-11 Western Blot-Three Pool 25 μ g of protein were run on SDS-PAGE. The subsequent nitrocellulose blot was probed with the following antibodies: A) Cadherin-11 monoclonal antibody; B) Pancadherin polyclonal antibody; C) β -catenin monoclonal antibody. The open arrow marks the location of the cadherin-11 band on the pancadherin blot.

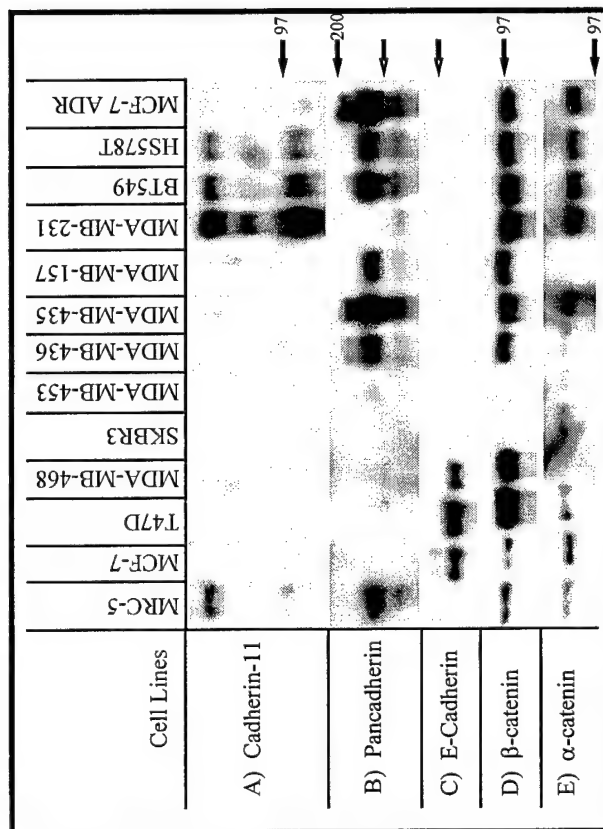
Figure 4. Cadherin-11 Immunocytochemistry Cells were seeded on coverslips and fixed in paraformaldehyde. The fixed cells were stained with a Cadherin-11 monoclonal antibody (A,B,D) or with normal mouse IgG (C). Secondary staining with appropriate Texas Red-labeled goat antibodies revealed cadherin-11 expression in BT549 cells (A) and HS578t cells (B), but not in MCF-7 cells (D). Cadherin-11 is expressed at sites of cell-cell contact, but also at lamellopodial extensions and possibly at regions of contact with the substrate. Staining with normal mouse IgG revealed no non-specific staining (C).

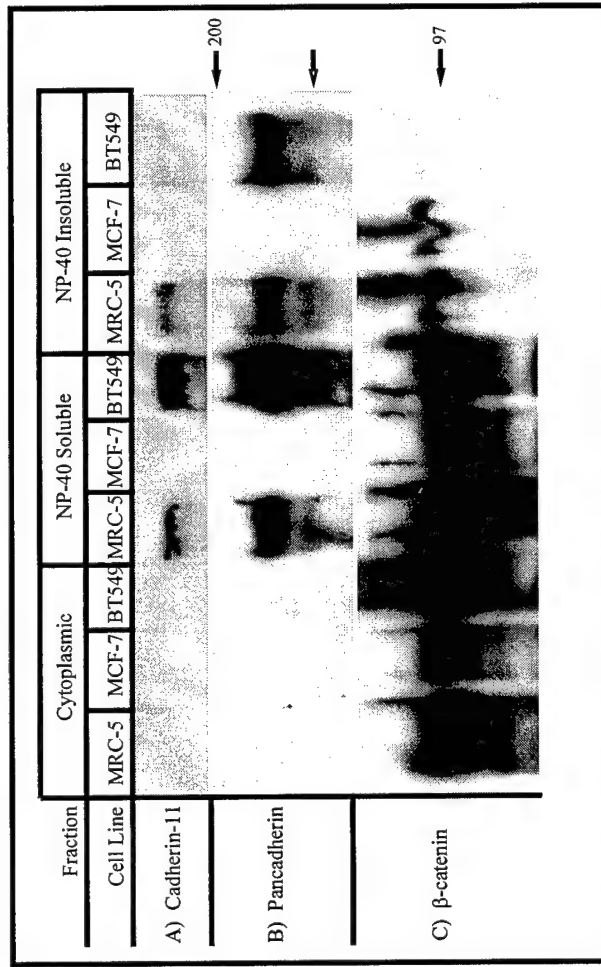
Figure 5. Immunoprecipitations Confluent cells were lysed in an NP-40 buffer and the lysate clarified by microcentrifugation. The lysate was pre-cleared with normal rabbit serum and protein-A sepharose. Next, 3 μ l of a polyclonal rabbit serum raised against either α -catenin (left) or β -catenin (right) were used for the immunoprecipitations. Immunoprecipitations with 10 μ g of normal rabbit IgG precipitated no immunoreactive bands (data not shown). The immunoprecipitation was run on SDS-PAGE and the subsequent blot was probed with the following antibodies: A) Cadherin-11 monoclonal antibody; B) Pancadherin polyclonal antibody; C) E-cadherin monoclonal antibody; D) α -catenin polyclonal; and E) β -catenin monoclonal antibody. The open arrow marks the location of the cadherin-11 band on the pancadherin blot.

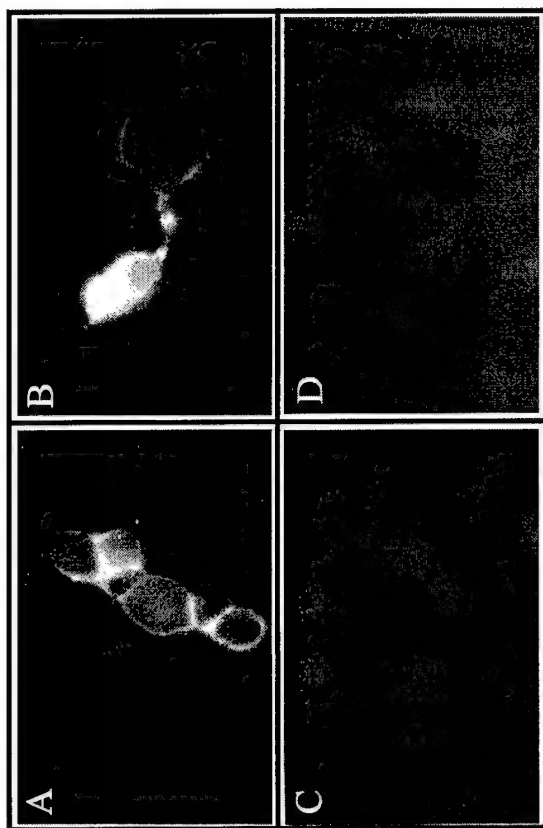


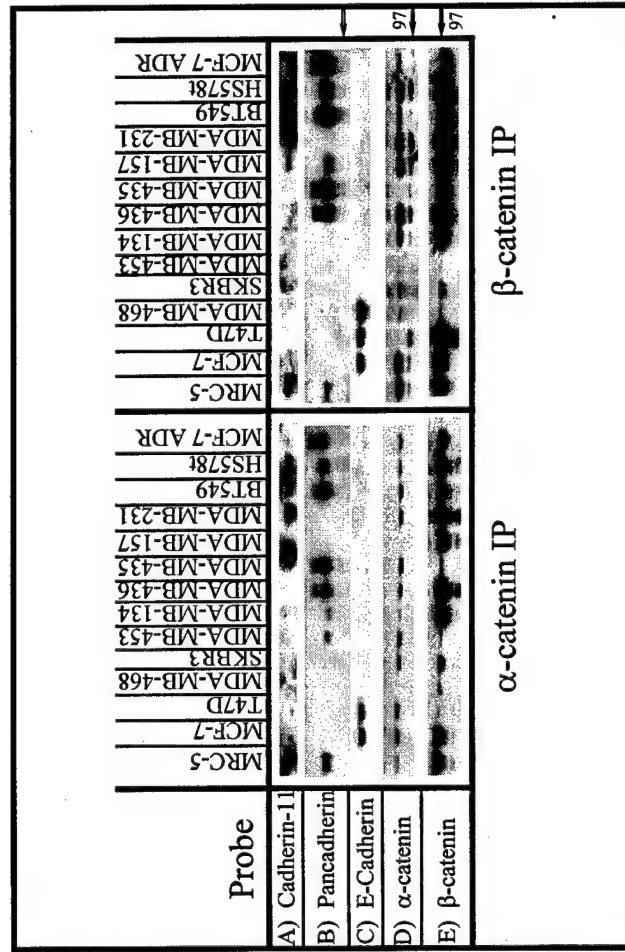












9-cis Retinoic Acid Regulates β -catenin/LEF Signaling

Michael J. Pishvaian[†], Vijayasurian Easwaran[†], and Stephen W. Byers*

The Lombardi Cancer Research Center and the Department of Cell Biology, Georgetown

University School of Medicine, Washington, D.C. 20007

[†]These Authors Contributed Equally

Contacts: *To whom correspondence should be addressed

Dr. Steve Byers, E412 The Research Building, Georgetown University Medical Center, 3970

Reservoir Road, NW Washington, DC 20007 Phone: (202) 687-1813 Fax: (202) 687-7505

Email: byersS@gunet.georgetown.edu

Running Title: RA Regulates β -catenin/LEF Signaling

Keywords: Retinoids, Retinoic Acid, β -catenin,

Lymphocyte Enhancer Binding Factor (LEF), Wnt,

Vitamin A derivatives (retinoids) are potent regulators of embryogenesis, cell proliferation, epithelial cell differentiation, and carcinogenesis. Some of the effects of retinoids in breast cancer cells are associated with changes in the cadherin- β -catenin adhesion and signaling system. In addition to its role in cell-cell adhesion, β -catenin is a member of the wnt signaling pathway, which regulates several developmental and neoplastic processes thought to be mediated by the β -catenin-induced activation of members of the TCF/LEF family of transcription factors. We now show that RA treatment reduces levels of cytoplasmic β -catenin and independently decreases LEF-reporter activity induced by exogenous or endogenous β -catenin. These results suggest that one mechanism whereby retinoic acid influences development, cell differentiation, and cancer is by directly or indirectly inhibiting the actions of β -catenin/LEF signaling.

1 Retinoic acid (RA), one of the more biologically active retinoids, inhibits the formation
2 of papillomas in the skin of mice and can prevent the transformation of mouse JB6 cells (1-3).
3 Retinoids also inhibit the growth of several human cancers, including melanoma, and colon,
4 breast, and prostate cancer (4-10). Retinoid action is mediated through retinoid receptor proteins,
5 RARs and RXRs, which can act to modulate gene expression, either directly by binding retinoic
6 acid responsive elements (RAREs) or indirectly by inhibiting the activity of other transcription
7 factor complexes such as AP-1 (11,12). RXR also functions through heterodimer formation with
8 other members of the steroid hormone receptor superfamily such as the thyroid hormone receptor
9 and the vitamin D receptor. However, many of the downstream pathways that mediate retinoid
10 induced changes in cells have yet to be defined.

11 β -catenin is a member of the wnt signaling pathway, and wnt signaling results in
12 increases in cytoplasmic and nuclear pools of β -catenin (13-16). Increases in cytoplasmic and
13 nuclear β -catenin are also associated with human colon cancer, melanoma, and medulloblastoma
14 and expression of a stable form of β -catenin can transform NIH 3T3 cells (17-20). Both
15 oncogenic and developmental effects of β -catenin are thought to be mediated by its interaction
16 with members of the TCF/LEF family of transcription factors (15-17).

17 Previous studies demonstrate a role for increased cell-cell adhesion and β -catenin
18 stability in mediating the effects of 9-cis RA on adhesion and differentiation (21,22). However,
19 these retinoid-induced increases in β -catenin appear to be in conflict with other results
20 demonstrating that elevated β -catenin levels and increased β -catenin stability are associated with
21 wnt signaling and LEF-mediated transactivation, as well as with oncogenesis (17-19,23). In
22 order to resolve this discrepancy, we first tested the ability of RA to influence the activity of a β -

catenin/LEF-regulated luciferase construct (24). SK-BR-3 cells, which express very low levels of β -catenin protein, were transfected with the LEF-reporter constructs and treated with RA (25,26). Despite the dramatic increases in total β -catenin protein levels, there was no increase in LEF-reporter activity (Fig. 1A). Since the signaling activity of β -catenin is likely mediated by its cytoplasmic pool we determined which pool of β -catenin was being influenced by RA (28). Figure 1B shows that RA increased the membrane and cytoskeletally-associated pools of β -catenin where β -catenin is able to interact with cadherins in a stable adherens junction, but RA did not increase the cytoplasmic pool of β -catenin.

In order to test if RA treatment could influence LEF-reporter activity in cells with elevated levels of cytoplasmic β -catenin, SK-BR-3 cells were transfected with β -catenin (29). As expected, LEF-reporter activity increased dramatically in β -catenin-transfected cells (Fig. 1A). Activity of a mutant LEF-reporter construct also increased slightly, a non-specific result of the very high level of β -catenin in the transiently transfected cells (Fig. 1A). Three pool fractionation revealed that the transfected β -catenin accumulated in the cytoplasm but not at the membrane (Fig. 1C). Treatment of β -catenin-transfected cells with RA dramatically decreased both cytoplasmic β -catenin and LEF-reporter activity but did not reduce levels of membrane-associated β -catenin (Fig 1A and C). Significantly, RA also decreased the cytoplasmic levels of the S37A constitutively stable form of β -catenin (Figure 1D)(30). We next examined the effects of RA on cells with higher levels of endogenous cytoplasmic β -catenin.

Elevated levels of endogenous cytoplasmic β -catenin and LEF-reporter activity are associated with the transformed phenotype in several cancers (17-19). Since RA inhibits the growth of several breast cancer cell lines, we were interested in determining the effects of RA on

cells which have constitutively elevated levels of cytoplasmic β -catenin and LEF-reporter activity (4,5). Figure 2A, shows the effects of RA on cytoplasmic β -catenin in several retinoid-responsive breast cancer cell lines. In two of these, MCF-7 and HS578T, RA reduces cytoplasmic levels of β -catenin (Fig. 2A), and LEF-reporter activity in a dose dependent manner (MCF-7, Fig. 2B; HS578T, data not shown). Considering the role of β -catenin in cell-cell adhesion, it is significant that those cell lines in which RA decreases β -catenin, RA also induces a morphological change and an apparent increase in cell-cell adhesion ((22), and data not shown). Figure 2C and D show that the reductions in cytoplasmic β -catenin occur in a time dependent manner with maximum reduction at 96 hours, which corresponds to the time frame for RA-induced decreases in cell proliferation (4). Significant reductions in LEF-reporter activity were apparent by 48 hours (MCF-7, Fig. 2E; HS578T, data not shown), but we were unable to determine the time course for maximum effect because LEF-reporter activity is lost after 72 hours (Data not shown). Nevertheless, these results suggest that RA treatment induces a cascade of events that result in reduced levels of cytoplasmic β -catenin and LEF-reporter activity.

Previous work has demonstrated that RA stabilizes cadherin-catenin interactions and can increase calcium-dependent cell-cell adhesion strength (21,22). In addition, cadherin overexpression can recruit β -catenin to the membrane and increase cell-cell adhesion strength while reducing levels of cytoplasmic β -catenin and inhibiting β -catenin signaling function in *Xenopus* (31,32). We wanted to determine whether the effects of RA on cytoplasmic β -catenin levels and LEF-reporter activity are dependent upon cadherin-mediated calcium-dependent cell-cell adhesion. Figure 3A shows that in SK-BR-3 cells the RA-mediated decrease in cytoplasmic β -catenin occurs only in the presence of calcium (33). Significantly, RA is able to reduce LEF-

1 reporter activity in the presence or absence of calcium (Fig 3B). Thus, while the RA-mediated
2 depletion of cytoplasmic β -catenin requires calcium-dependent adhesion, the ability of RA to
3 inhibit LEF-reporter activity does not.

4 In colon cancer cells the levels of cytoplasmic β -catenin are regulated by the product of
5 the tumor suppressor protein, adenomatous polyposis coli, APC (34,35). We next wanted to test
6 if the actions of RA on β -catenin/LEF signaling required APC. Figure 4A shows that RA
7 effectively inhibits LEF-reporter activity in retinoid-sensitive APC-mutant colon cancer cells
8 (CaCo-2) and demonstrates that APC is not required for RA to inhibit β -catenin/LEF signaling.
9 Significantly the cytoplasmic pool of β -catenin in CaCo-2 cells, as well as in two other colon
10 cancer cell lines is unaffected by RA (Fig. 4B). If the actions of RA involved the targeting of
11 cytoplasmic β -catenin for ubiquitination and proteosomal degradation (in an APC-independent
12 manner) one would anticipate that the signaling activity of stable, non-ubiquitinatable mutants of
13 β -catenin would not be affected by RA. In contrast, figure 4C shows that RA effectively
14 inhibits LEF-reporter activity induced by the S37A stable mutant form of β -catenin and further
15 indicates that the effects of RA are unrelated to events which regulate β -catenin ubiquitination
16 and proteosomal degradation. These data point to a direct effect of RA on the regulation of
17 nuclear β -catenin/LEF-mediated transactivation.

18 Retinoids are important signaling molecules both in the adult and in the developing
19 embryo. RA can dramatically affect pattern formation, effects that are in part mediated by
20 members of the Hox gene family and by segment polarity genes such as sonic hedgehog (36).
21 Wnt and its *Drosophila* homologue wingless are also important in the establishment of
22 patterning in embryos, and overexpression of wnt-1 or β -catenin in *Xenopus* results in axis

1 duplication (13-16). Our demonstration that RA influences β -catenin/LEF-signaling suggests
2 that these two important developmental pathways may interact more directly than previously
3 envisioned. The significance of β -catenin in cancer is less well defined. β -catenin is a
4 component of the adherens junction, which can act as a tumor suppressor (37,38). However,
5 elevated cytoplasmic and nuclear β -catenin may also be oncogenic. Retinoids are known to be
6 potent antitumor agents, but it is still unclear how retinoids prevent cancer. Because increased
7 levels of cytoplasmic β -catenin and/or increased LEF-signaling may result in cellular
8 transformation, we propose that one mechanism whereby RA acts to inhibit or reverse
9 tumorigenesis may be by reducing β -catenin/LEF-transactivation.

10 Since the effects of RA are mediated via activated nuclear receptors, it is likely that the
11 mechanism of inhibition of LEF-promoter activity is secondary to the activation of retinoid
12 receptors. Several non-exclusive scenarios can be envisioned: 1) Retinoid regulated gene
13 products could promote the decrease in cytoplasmic β -catenin which in turn would reduce LEF-
14 transactivation. The decrease in cytoplasmic β -catenin could be due to a decrease in the stability
15 of the cytoplasmic pool of β -catenin and/or to a removal of β -catenin from the cytoplasm,
16 perhaps by an increase in cadherin or α -catenin expression. However, the demonstration that
17 low calcium medium can prevent the retinoid-induced decrease in cytoplasmic β -catenin but
18 does not affect retinoid-induced reductions in LEF-reporter activity indicates that these two
19 effects are independent; 2) Retinoid regulated gene products could inhibit β -catenin/LEF-
20 transactivation directly; 3) Activated retinoid receptors could inhibit β -catenin/LEF-
21 transactivation directly, or indirectly through competition for common co-factors such as occurs
22 between retinoid receptors and AP-1 (12).

1 The inhibition of β -catenin/LEF signaling strongly suggests that 9-cis RA and probably other
2 retinoids should be considered as therapeutic agents for cancers in which β -catenin is
3 overexpressed and β -catenin/LEF-transactivation is overactive. It is also significant that PPAR
4 ligands influence the degree of polyposis found in animal models of colon cancer, a disease that
5 results from activated β -catenin/LEF signaling (39-41). PPARs need to heterodimerize with
6 RXR in order to function (42). Consequently, it is possible that the effects of PPAR ligands are
7 directly mediated by changes in β -catenin/LEF signaling. Taken together, these results show that
8 treatment of cells with 9-cis retinoic acid increase the adhesive function of β -catenin and
9 decrease its signaling activity. Because changes in cadherin/catenin-based cell-cell adhesion and
10 β -catenin/LEF signaling are important in carcinogenesis and in many aspects of development,
11 these results point to a novel and perhaps general mechanism whereby retinoids can both
12 increase epithelial differentiation and decrease cell proliferation.

1 Bibliography

- 2 1. J. J. Li, Z. Dong, M. I. Dawson, N. H. Colburn, *Cancer Res.* **56**, 483 (1996).
- 3 2. A. K. Verma, C. J. Garcia, C. L. Ashendal, R. K. Boutwell, *Cancer Res.* **43**, 3045 (1983).
- 4 3. A. K. Verma, T. J. Slaga, P. W. Wertz, G. C. Mueller, R. K. Boutwell, *Cancer Res.* **40**, 2367
- 5 (1980).
- 6 4. P. Fitzgerald, M. Teng, R. A. Chandraratna, R. A. Heyman, E. A. Allegretto, *Cancer Res.* **57**,
- 7 2642 (1997).
- 8 5. S. Toma, L. Isnardi, P. Raffo, G. Dastoli, *Int. J. Cancer* **70**, 619 (1997).
- 9 6. S. E. Fliegel, *et al*, *Journal of Cutaneous Pathology* **19**, 27 (1992).
- 10 7. M. Edward, J. A. Gold, R. M. Mackie, *Clin. Exp. Metas* **10**, 61 (1992).
- 11 8. N. M. Housein, D. E. Brattain, M. K. McKnight, *Cancer Letters* **40**, 219 (1988).
- 12 9. P. J. O'Dwyer, T. S. Ravikuma, D. P. McCabe, G. J. Steele, *Journal of Surgical Research* **43**,
- 13 550 (1987).
- 14 10. S. E. Blutt, E. A. Allegretto, J. W. Pike, N. L. Weigel, *Endocrinology* **138**, 1491 (1997).
- 15 11. L. J. Gudas, M. B. Sporn, A. B. Roberts, *The Retinoids: Biology, Chemistry and Medicine*,
- 16 M.B. Sporn, A.B. Roberts and D.S. Goodman, Eds. (Raven Press, New York, 1994), p. 443.
- 17 12. R. Schule, *et al*, *Proc. Natl. Acad. Sci.* **88**, 6092 (1991).
- 18 13. N. Funayama, F. Fagotto, P. McCrea, B. M. Gumbiner, *J. Cell Biol.* **128**, 959 (1995).
- 19 14. K. A. Guger, B. Gumbiner, *Dev. Biol.* **172**, 115 (1995).
- 20 15. M. Molenaar, *et al*, *Cell* **86**, 391 (1996).
- 21 16. J. Behrens, *et al*, *Nature* **382**, 638 (1996).
- 22 17. P. J. Morin, *et al*, *Science* **275**, 1787 (1997).

- 1 18. B. Rubinfeld, *et al*, *Science* **275**, 1790 (1997).
- 2 19. R. H. Zurawel, S. H. Chiappa, C. Allen, C. Raffel, *Cancer Res.* **58**, 896 (1998).
- 3 20. I. Whitehead, H. Kirk, K. Kay, *Mol. Cell Biol.* **15**, 704 (1995).
- 4 21. S. Byers, *et al*, *Endocrinology* **137**, 3265 (1996).
- 5 22. S. J. Vermeulen, E. A. Bruyneel, F. M. Van Roy, M. M. Mareel, M. E. Bracke, *Br. J. Cancer*
- 6 **72**, 1447 (1995).
- 7 23. J. Papkoff, B. Rubinfeld, B. Schryver, P. Polakis, *Mol. Cell Biol.* **16**, 2128 (1996).
- 8 24. V. Korinek, *et al*, *Science* **275**, 1784 (1997).
- 9 25. Cell Culture and Retinoid Treatment. Breast cancer cell lines cells were grown in DMEM
- 10 (Gibco) supplemented with 5% FBS and antibiotics. Cells were treated with 1 μ M 9-*cis* Retinoic
- 11 Acid or Ethanol for 96 hours (SK-BR-3 cells, 48 hours).
- 12 26. Transfections and Luciferase Assays. For luciferase assays: Cells were seeded in 12 well
- 13 plates at 1×10^5 cells per well. Cells were transiently transfected with 1 μ g of the LEF-reporter
- 14 pTOPFLASH (optimal motif) or pFOPFLASH (mutant motif) (24); with 0.02 μ g of pCMV-
- 15 Renilla Luciferase (Promega); and with 5 μ g pCDNA3 (Invitrogen) or pCDNA3/WT β -catenin
- 16 (30). The calcium phosphate method was used (27). RA treatment was initiated 24 hours post-
- 17 transfection. Luciferase activity was monitored using the DUAL-Luciferase Assay System
- 18 (Promega). The experimental LEF-luciferase reporter activity was controlled for transfection
- 19 efficiency by comparison with the constitutively expressed Renilla Luciferase. For three pool
- 20 fractionation: Subconfluent cells in a 10 cm dish were transfected with 25 μ g of either the
- 21 pCDNA3 empty vector (Invitrogen) or pCDNA3/WT β -catenin by the calcium-phosphate

method (27),(30). Retinoid treatment of transfected cells was initiated 24 hours post-transfection.

27. J. Sambrook, E. Fritsch, T. Maniatis, *Molecular cloning: A laboratory manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1989).

28. Cellular Subfractionation and Western Blots. Cells from confluent 10 cm dishes were isolated and dounce homogenized in a hypotonic solution (10 mM Tris, 0.2 mM MgCl₂, pH 7.5).

The homogenate was centrifuged first for 10 min. at 3000 X g, to remove nuclei. The supernatant was then centrifuged at 150,000 X g for 1 hr. The supernatant of this centrifugation,

defined as the cytoplasmic fraction, was added to 4 volumes of ethanol and the proteins precipitated overnight. The proteins were then collected by ultracentrifugation and solubilized in

sample buffer (2% SDS, 60 mM Tris, pH 6.8, 10% glycerol). For total membrane isolates, the pellet from the first ultracentrifugation was solubilized directly in sample buffer. For three pool

fractionation, the pellet from the first ultracentrifugation was solubilized in a 1% Nonidet P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 8.0) for 30 min., and clarified in a

microcentrifuge for 15 min. The resulting supernatant is the NP-40-soluble membrane fraction.

The pellet is the NP-40-insoluble fraction, representing cytoskeletally associated proteins. Both were solubilized in sample buffer. Protein content in the samples was measured (Bio-Rad).

Proteins were separated on an 8% reducing polyacrylamide minigel (Novex), transferred to nitrocellulose (Protran) and blocked overnight in 5% milk. The blot was then probed with a

monoclonal antibody to β -catenin (monoclonal antibody, Transduction Labs).

- 1 29. β -catenin constructs. A Wild type and a S37A, degradation-resistant mutant construct of β -
- 2 catenin was developed by Keith Orford (30). Both constructs were synthesized in pCDNA3 and
- 3 are flag and HA-tagged.
- 4 30. K. Orford, C. Crockett, J. P. Jensen, A. M. Weissman, S. W. Byers, *JBC* **272**, 24735 (1997).
- 5 31. J. Heasman, *et al*, *Cell* **79**, 791 (1994).
- 6 32. F. Fagotto, N. Funayama, U. Gluck, B. M. Gumbiner, *J. Cell Biol* **132**, 1105 (1998).
- 7 33. For low calcium treatments: SK-BR-3 cells were plated and transfected in normal DMEM
- 8 with CaCl_2 . Following transfection and glycerol shock, the cells were washed 3 X in PBS
- 9 without CaCl_2 . The cells were then grown for 48 hours +/- RA in DMEM without CaCl_2 ,
- 10 supplemented with either 2 mM or 50 μM CaCl_2 .
- 11 34. S. Munemitsu, I. Albert, B. Souza, B. Rubinfeld, P. Polakis, *Proc. Natl. Acad. Sci. U. S. A.*
- 12 **92**, 3046 (1995).
- 13 35. B. Rubinfeld, I. Albert, E. Porfiri, S. Munemitsu, P. Polakis, *Cancer Res.* **57**, 4624 (1997).
- 14 36. L. J. Gudas, *JBC* **269**, 15399 (1994).
- 15 37. M. Takeichi, *Curr Opin Cell Bio* **5**, 806 (1993).
- 16 38. M. Takeichi, *Annu Rev Biochem* **59**, 237 (1990).
- 17 39. A. M. Lefebvre, *et al*, *Nature Med.* **4**, 1053 (1998).
- 18 40. P. Sarraf, *et al*, *Nature Med.* **4**, 1046 (1998).
- 19 41. E. Saez, *et al*, *Nature Med.* **4**, 1058 (1998).
- 20 42. B. M. Forman, J. Chen, R. M. Evans, *Ann. N. Y. Acad. Sci.* **804**, 266 (1996).

1 *Acknowledgments: We would like to thank Marc van der Wetering (Department of Immunology,*
2 *University Hospital, Utrecht, The Netherlands) for providing the TOP and FOPFLASH LEF-*
3 *reporter vectors.*

4 *This work was funded by a grant from the U.S. Department of Defense. MJP and VE are*
5 *also recipients of predoctoral fellowships from the Department of Defense.*

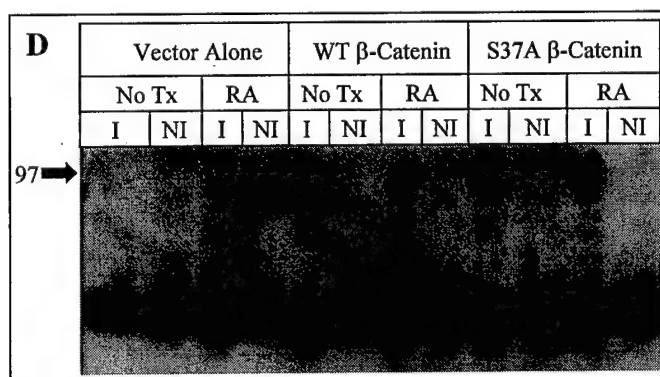
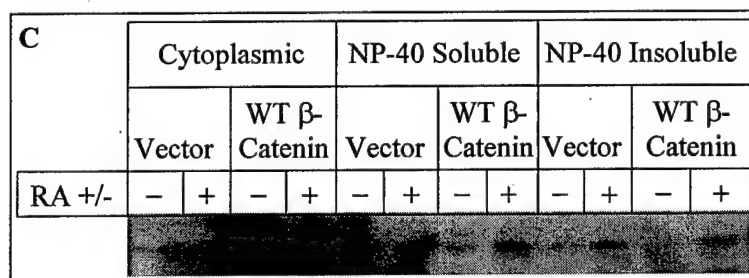
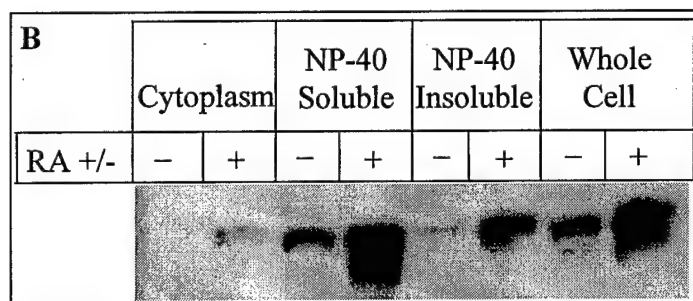
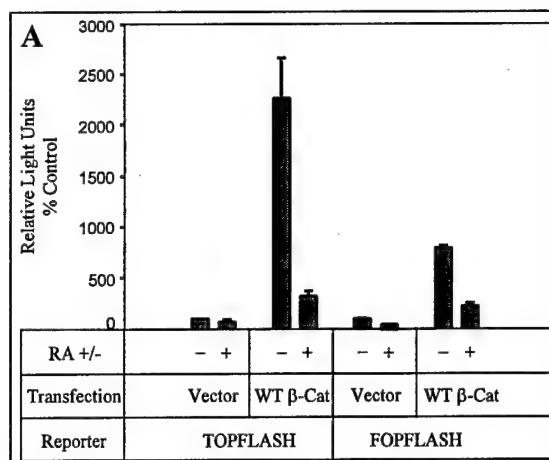
Figure 1: RA decreases LEF-reporter activity and levels of exogenous cytoplasmic β -catenin in SK-BR-3 cells. 1A) Despite the increase in levels of β -catenin induced by RA, there is no corresponding increase in levels of LEF-reporter activity (TOPFLASH) with the vector alone. Exogenous expression of β -catenin dramatically increases LEF-reporter activity, and RA reduces LEF-reporter activity almost to baseline levels. Activity of a mutant LEF-reporter construct also increased slightly, a non-specific result of the very high level of β -catenin in the transiently transfected cells. 1B) SK-BR-3 cells were fractionated into 3 pools. RA increases the levels of β -catenin only in the NP-40-soluble and -insoluble pools, and not in the cytoplasm. 1C) SK-BR-3 cell were transiently transfected with a β -catenin construct, or with the empty vector then fractionated into 3 pools. The transfected β -catenin is found predominantly in the cytoplasm. RA treatment decreases cytoplasmic levels of β -catenin while still increasing levels in the soluble and insoluble pools. 1D) RA was also able to decrease levels of the S37A mutant β -catenin. Cell lysates of transfected SK-BR-3 cells were immunoprecipitated with an anti-flag monoclonal antibody (Immune lanes) or with mouse IgG (Non-immune lanes) and analyzed by western blot with a monoclonal antibody to β -catenin. Levels of the S37A mutant are higher than the wild type, yet RA is able to reduce the levels of both the wild type and mutant.

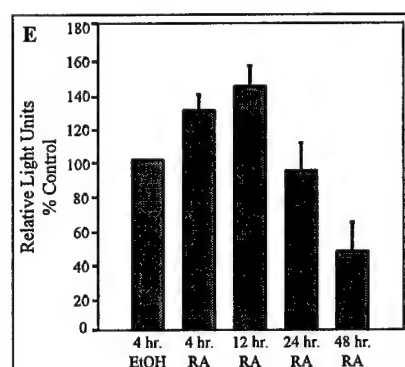
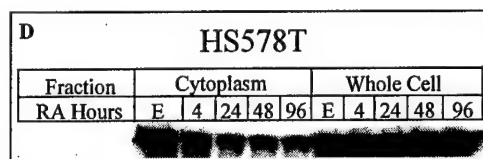
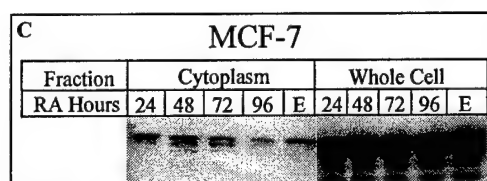
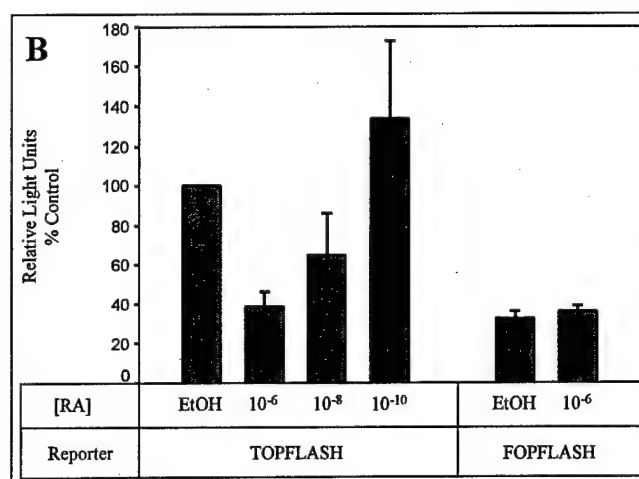
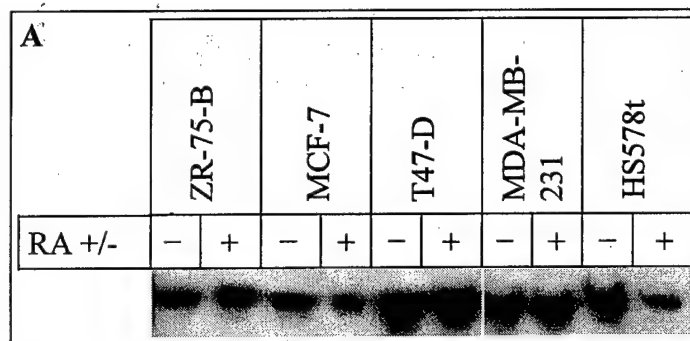
Figure 2: RA reduces endogenous levels of cytoplasmic β -catenin and decreases LEF-reporter activity. 2A) Cytoplasmic levels of β -catenin were compared in four RA-responsive cell lines, ZR-75-B, MCF-7, T47D, and HS578T, and in one unresponsive cell line, MDA-MB-231. RA reduces levels of β -catenin in MCF-7 and HS578T cells. 2B) RA reduces LEF-reporter activity in MCF-7 cells in a dose dependent manner, but has no effect on pFOPFLASH activity.

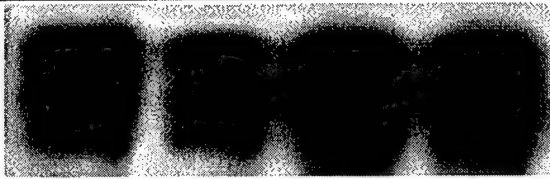
2C and D) RA-induced reductions in cytoplasmic β -catenin in MCF-7 cells (Fig. 2C) and HS578T cells (Fig. 2D) were apparent by 24 hours, but were maximal at 96 hours. 2E) RA-induced reductions in LEF-reporter activity in MCF-7 cells was apparent by 48 hours. Luciferase assays could not be performed after 96 hours because transient luciferase expression would be lost within 72 hours.

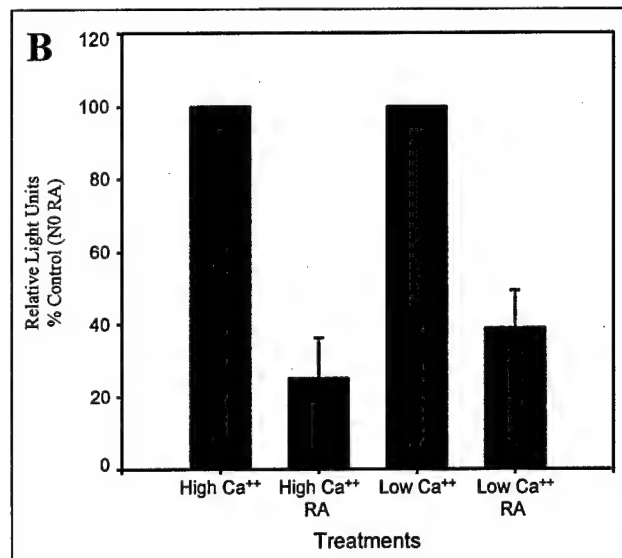
Figure 3: The RA-mediated reduction in cytoplasmic β -catenin requires calcium while the reduction in LEF-reporter activity does not. 3A) In SK-BR-3 cells transfected with wild type β -catenin, RA reduces levels of cytoplasmic β -catenin in cells grown in 2 mM calcium, but not in cells grown in 50 μ M calcium. 3B) In contrast, RA is able to reduce LEF-reporter activity in cells grown in both 2 mM and 50 μ M calcium.

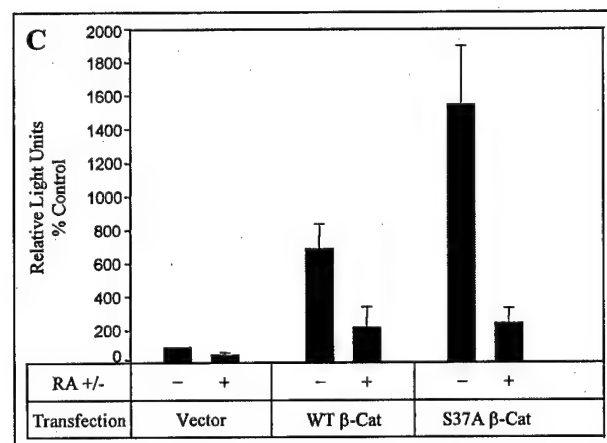
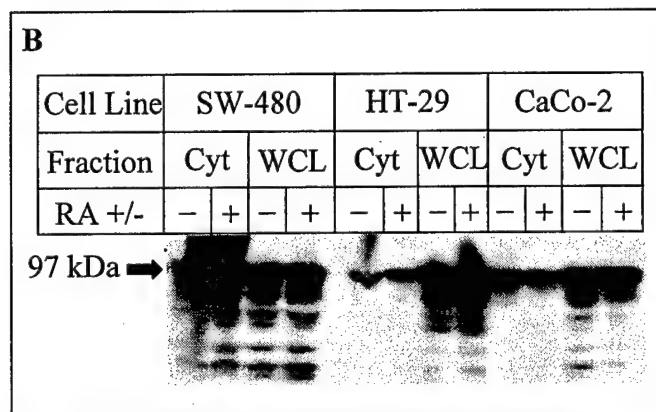
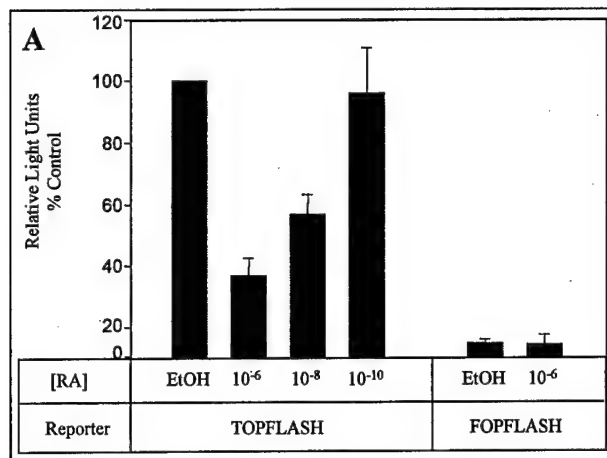
Figure 4: RA reduces β -catenin/LEF-reporter activity independent of APC activity and ubiquitin-proteasome mediated degradation of β -catenin. 4A) In CaCo-2 cells, which are retinoid responsive, but lack a functional APC gene, RA reduces LEF-reporter activity in a dose dependent manner, but has no effect on pFOPFLASH activity. 4B) However, RA is unable to reduce levels of cytoplasmic β -catenin in CaCo-2 cells, and in two other colon cancer cells lines, SW-480 and HT-29, both of which also lack a functional APC gene. 4C) RA is also able to reduce LEF-reporter activity in SK-BR-3 cells induced by exogenous expression of the S37A degradation resistant mutant of β -catenin.





A	2 mM Calcium		50 μ M Calcium		
	RA +/-	-	+	-	+
					





1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

β -catenin regulates contact inhibition, anchorage independent growth, anoikis and radiation-induced cell cycle arrest

Keith Orford, Caroline C. Orford, and Stephen W. Byers*

The Lombardi Cancer Research Center and the Department of Cell Biology, Georgetown University School of Medicine, Washington, DC 20007

Contacts: * To whom correspondence should be addressed:

Dr. Stephen Byers, E415 The Research Building, GUMC, 3970 Reservoir Rd., N.W.
Washington, DC 20007 Phone: (202) 687-1813 Fax: (202) 687-7505
E-mail: byerss@gunet.georgetown.edu

Running Title: β -catenin Transforms a Normal Epithelial Cell Line

Keywords: β -catenin, cell transformation (neoplastic), contact inhibition, cell cycle, apoptosis

- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13

3 β -catenin is an important regulator of cell-cell adhesion and embryonic
 4 development that associates with and regulates the function of the LEF/Tcf family of
 5 transcription factors. Mutations of β -catenin and the tumor suppressor gene APC occur
 6 in human cancers but it is not known if and by what mechanism increased β -catenin
 7 causes cellular transformation. This study demonstrates that modest over-expression of
 8 β -catenin in a normal epithelial cell results in cellular transformation. These cells form
 9 colonies in soft agar, survive in suspension, and continue to proliferate at high cell
 10 density and following γ -irradiation. Endogenous cytoplasmic β -catenin levels and
 11 signaling activity were also found to oscillate during the cell cycle. Taken together, these
 12 data point to a role of β -catenin in the regulation of the G₁ to S phase transition and
 13 suspension-induced apoptosis (anoikis).

Introduction

β -catenin is a 92kDa protein associated with the intracellular tail of the intercellular adhesion molecule E-cadherin (Ozawa et al., 1989). Through this association, β -catenin plays an important role in strong cell-cell adhesion as it links E-cadherin (and other members of the cadherin family) to the actin cytoskeleton through the protein α -catenin (Hirano et al., 1992; Kemler, 1993). One mechanism by which cell-cell adhesion can be negatively regulated is via the phosphorylation of β -catenin on tyrosine residues (Behrens et al., 1993). There are some indications that this may be an important event in the transition from a benign tumor to an invasive, metastatic cancer (Sommers et al., 1994).

β -catenin is also a regulator of embryogenesis, a role that was first suspected when it was shown to be the mammalian homolog of the *Drosophila* segment polarity gene *armadillo* (Peifer et al., 1992). Further studies in *Drosophila* and *Xenopus* have revealed that β -catenin is a component of the highly conserved Wnt/Wingless signal transduction pathway which regulates body patterning in both species (Peifer, 1995; Gumbiner, 1997).

The membrane-associated and cytoplasmic pools of β -catenin have disparate activities: adhesion and signaling, respectively. The accumulation of cytoplasmic β -catenin drives its interaction with a member of the LEF/Tcf family of nuclear transcription factors which results in altered gene expression, that is the transduction of the Wnt/Wg signal (Clevers and van de Wetering, 1997). This accumulation of

cytoplasmic β -catenin is, at least in part, regulated at the level of its degradation (Peifer et al., 1994; Peifer, 1995; Papkoff et al., 1996). In the absence of the Wnt/Wg signal, phosphorylation of specific serine residues on β -catenin leads to its ubiquitination and degradation, removing it from the cytoplasm (Orford et al., 1997). Mutations of these serine residues inhibit the ubiquitination of β -catenin, which causes it to accumulate and signal constitutively (Orford et al., 1997; Morin et al., 1997).

Along with its position in a growth factor signaling pathway, the demonstration of an interaction between β -catenin and the product of the tumor suppressor gene Adenomatous Polyposis Coli (APC) suggest that it is involved in oncogenesis (Rubinfeld et al., 1996). Tumor cell lines with a loss of one copy of APC and harboring mutations in the other allele have high levels of cytoplasmic (signaling) β -catenin which is markedly reduced when functional APC is re-introduced (Munemitsu et al., 1995). Importantly, all mutant forms of APC found in human cancers are unable to reduce β -catenin levels in these cells. The importance of elevated β -catenin in human cancer was further substantiated when mutations in the β -catenin gene were described in colon cancer and melanoma cell lines (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997). At least one of these mutations results in a more stable form of the protein.

A retroviral insertion screen for oncogenes using the NIH-3T3 cell line also implicated β -catenin as a possible oncogene as the insertion of the retrovirus resulted in the expression of a β -catenin protein which lacked the N-terminus (Whitehead et al., 1995). In contrast, over-expression of a stabilized form of β -catenin is unable to mimic the morphological effects of Wnt-1 in fibroblasts (Young et al., 1998).

Although much is now known about this signaling system, the actual cellular processes in which β -catenin plays a regulatory role is unclear. As described above, it regulates cadherin-mediated cell-cell adhesion. Although it appears to regulate gene expression, few target genes have been demonstrated. Based on its relationship with Wnt and APC, it is possible that β -catenin may positively regulate cellular proliferation or inhibit apoptosis. It is also tempting to speculate that the adhesive and the putative oncogenic functions of β -catenin are related and that it may be, at least in part, the mechanistic link between cell-cell adhesion, contact inhibition and/or apoptosis. However, no studies have directly tested the hypothesis that β -catenin is actually oncogenic.

This report utilizes the MDCK cell line to determine the impact of over-expressing wild-type or a stabilized mutant form of β -catenin in non-transformed epithelial cells. The data demonstrate that β -catenin alters cell cycle progression and confers enhanced growth in soft agar, a surrogate marker for tumorigenicity. In addition, β -catenin confers resistance to suspension-mediated apoptosis (anoikis) and radiation damage and allows cells to continue cycling when cultured at confluence. In short, β -catenin transforms normal epithelial cells in culture.

Materials and Methods

Cells, Plasmids, and Stable Transfections: MDCK cells are a canine kidney-derived non-transformed epithelial cell line that are maintained in DMEM (Gibco) supplemented with 5% fetal bovine serum (FBS). A1N4 cells are a human mammary non-transformed

1 epithelial cell line that are grown in IMEM supplemented with 0.5% FBS, 0.5%
2 hydrocortisone, 5 µg/ml insulin, and 10 ng/ml epidermal growth factor (EGF) (Stampfer
3 and Bartley, 1988). These cells synchronize in G₀ in the absence of EGF. The wild-type
4 (WT) and S37A mutant (S37A) β-catenin plasmids were described previously (Orford et
5 al., 1997). The bacterial chloramphenicol acetyltransferase gene driven by the CMV
6 promoter of the pcDNA 3 plasmid (Invitrogen) served as the negative control (CON).
7 For stable transfections, 800,000 MDCK cells were plated per 100 mm tissue culture
8 plate. The next day, 15 µg of the various plasmids were transfected using the
9 Lipofectamine PLUS method (Gibco); 32 µl Lipofectamine and 45 µl PLUS reagent. 48
10 hours later the cells were split 1:20 and cultured for two weeks in the presence of 500
11 µg/ml of Geneticin (Gibco). An approximately equal number of colonies grew up for
12 each transfected plasmid. For each transfection, all of the colonies were trypsinized and
13 combined to give pooled stable cell lines.

14
15 Immunoblotting, Immunofluorescence, and Antibodies: *Immunoblotting-* Whole cell and
16 cytoplasmic lysates were made and immunoblotting performed as described previously
17 (Orford et al., 1997).

18 *Immunofluorescence-* Cells were grown to confluence in 4-well BIOCOAT
19 chamber slides (Falcon). Cells were washed twice in PBS and fixed in 4%
20 paraformaldehyde in PBS for ten minutes. Cells were then permeablized in 0.2% Triton
21 X-100, 4% paraformaldehyde in PBS for ten minutes. After washing in PBS, cells were
22 blocked in 3% ovalbumin for 1 hour. The chambers were incubated with primary

1 antibodies overnight at 4°. After 5x5min washes in PBS, secondary antibody was added
2 for one hour. Primary and secondary antibodies were diluted in 6% normal goat serum.
3 After removal of the secondary antibody, the chambers were washed 5x5min in PBS and
4 the chambers removed. The cells were mounted with Vectashield (Vector).

5 *Antibodies-* the anti- β -catenin (C19220) and anti-p27 (K25020) MAbs were from
6 Transduction Laboratories. The anti-HA MAb (HA-11) was purchased from Berkeley
7 Antibody Company (BAbCo). The anti-E-cadherin (SHE78-7) MAb was purchased from
8 Zymed. Peroxidase and fluorescein-labeled secondary antibodies were purchased from
9 Kirkegaard and Perry.

10

11 β -catenin/LEF Signaling Assays: In 12-well dishes, cells were transfected with 0.5 μ g of
12 the TOPFLASH LEF reporter plasmid (van de Wetering et al., 1997) and 0.005 μ g of the
13 constitutively expressed Renilla luciferase as a normalization control. The cells were
14 lysed and assayed for Firefly and Renilla luciferase activities using the STOP & GLO
15 assay (Promega). All results are normalized to the Renilla luciferase activity.

16

17 Soft Agar Growth Assay: For each cell line, 150,000 cells were suspended in 3 mls
18 DMEM + 5% FBS and warmed to 37°C. 300 μ l of a pre-warmed (52°C) 3% agarose/PBS
19 solution was mixed with the cell suspension and then layered into 3 wells of a six well
20 plate (1 ml/well) which were previously coated with 1 ml of 0.6% agarose in DMEM.
21 The agar was allowed to solidify at room temperature for 20 minutes before 3 mls of
22 growth medium was added to each well. The medium was changed every three days.

1 After fourteen days, the colonies were counted by an Omnicon 3600 Colony Counter and
2 photographed.

3
4 Assays of cell proliferation: 15,000 cells were plated into 60 mm dishes. At each time
5 point, the cells were washed once in PBS and trypsinized in 1 ml trypsin/versene (Gibco).
6 The single cell suspension was counted on a Coulter Counter set at 10 μ m min. and 20
7 μ m max. diameter. Each data point was performed in triplicate. Means and standard
8 error were calculated and graphed using Sigmaplot.

9
10 Plating Efficiency Assay: For each cell line, 100 cells were plated onto each of three 100
11 mm tissue culture dishes in DMEM+ 5% FBS. Four days after plating the colonies were
12 photographed at 400X magnification. After eight days the cells were washed with PBS,
13 stained with crystal violet and washed with water. The colonies were counted and then
14 photographed. The plating efficiency is the mean number of colonies per dish/100 cells
15 plated per dish.

16
17 Quantification of cell shedding: Cells were cultured in 6 well plates three days post-
18 confluence. The cells were washed two times in PBS and 2 mls fresh medium added to
19 each well. 24 hours later, the shed cells were removed with medium, and counted on a
20 Coulter Counter as described above.

21
22 Cell cycle analyses: Two flow cytometric assay were used. *Vindelov method*- Cells were

1 washed in PBS and trypsinized. Cells were washed in PBS and pelleted. After removing
2 wash buffer, the pellet was vortexed and resuspended in 0.1 ml of Citrate/DMSO buffer
3 (250 mM sucrose, 40 mM trisodium citrate-2H₂O, 5% DMSO, pH 7.60). The pellets are
4 then frozen at -80°C. The cells are then processed as in (Vindelov et al., 1983).

5 *Ethanol fixation method-* Cells were washed once in PBS and trypsinized.
6 Trypsinized cells were pelleted at 1000x g and washed in 5 mls cold PBS. Following a
7 second centrifugation, the cells were resuspended in 0.5 ml cold PBS and fixed by
8 dripping in 1.5 mls cold 100% ethanol while slowly vortexing the cell suspension. After
9 at least one hour at 4°C, the cells were stained with propidium iodide and DNA content
10 measured by flow cytometry. The ethanol fixation method was also used for the flow
11 cytometric analysis of apoptosis.

12
13 Cell synchronization experiments: *β-catenin protein level-* A1N4 cells were plated in
14 100 mm tissue culture dishes and grown overnight to approximately 40% confluency.
15 The cells were washed three times in PBS and then maintained in the absence of EGF for
16 46 -50 hours. This synchronized over 95% of the cells in the G₀/G₁ phase of the cell
17 cycle. To stimulate re-entry into the cell cycle, EGF-containing medium was added back
18 to the cells. Parallel dishes were analyzed at each time point for β-catenin protein (whole
19 cell or the cytoplasmic pool) and for the cell cycle distribution.

20 *β-catenin/LEF signaling-* 50,000 A1N4 cells were plated per well of 12-well
21 dishes and transfected with 1 μg of the TOPFLASH reporter plasmid and 0.01 μg of the
22 Renilla control plasmid by the calcium phosphate method. The cells were then

1 synchronized by EGF starvation (G_0/G_1) or 1 μ M nocodazole (G_2/M) or treated with the
2 proteosomal inhibitor ALLN which stabilizes β -catenin. The cells were collected and the
3 luciferase measurements were made as described previously.

4
5 Anoikis assay: Confluent cells were trypsinized into a single cell suspension. 700,000
6 cells were plated in 150mm tissue culture dishes coated with 0.8% agarose to which they
7 could not attach. At the various time points the cells were collected, washed in PBS, and
8 any cell aggregates were dispersed by trypsinization. Cells were then analyzed for
9 apoptosis in one of two ways. Some samples were analyzed by flow cytometry as above
10 (Cell cycle analyses, ethanol fixation). In this analysis, the hypodiploid peak constituted
11 the apoptotic population. The other samples were stained with fluorescein-labeled
12 Annexin-V and propidium iodide (Trevigen) according to the manufacturer's protocol
13 and analyzed by flow cytometry.

14
15 γ -irradiation: In order to obtain equal numbers of plated cells, 750,000 CON and 500,000
16 WT and S37A cells were plated in T75 tissue culture dishes (See below). 26 hours later
17 the flasks were exposed to 5 Gy of γ -irradiation. Another group of flasks received a
18 mock irradiation (0 Gy). At 8hr. and 24 hr. post-irradiation, the cells were trypsinized
19 and their cell cycle profile was determined.

20 21 **Results**

22 *Expression of β -catenin transgenes in MDCK cells*

In order to investigate the effects of β -catenin on normal cellular function, MDCK cells were stably transfected with constitutively expressed β -catenin transgenes that have been engineered to contain a carboxy-terminal hemagglutinin (HA) epitope tag. In addition to WT β -catenin, a construct harboring a previously described serine to alanine point mutation at residue 37 (S37A) was utilized which encodes for a β -catenin protein largely resistant to ubiquitination (Orford et al., 1997). The cell lines are pooled stable transfectants, that is, after selection with G418, all of the drug resistant colonies resulting from each transfection were combined. As a negative control, a cell line expressing the bacterial chloramphenicol acetyl transferase gene was generated (CON). Pooled stable cell lines were generated in order to avoid the phenotypic artifacts that can result from the selection and propagation of individual clones derived from single transfected cells. At the level of immunoblotting and immunofluorescence, expression of the HA tag was detectable only in the cell line expressing the more stable S37A mutant (Fig. 1B). We believe that the C-terminus of β -catenin is proteolytically processed as we have encountered considerable difficulty detecting this HA tag unless it is very highly expressed. In fact, C-terminal cleavage of β -catenin has been demonstrated under certain circumstances (Brancolini et al., 1997). Whole cell lysates do not exhibit any significant increase in total β -catenin levels (data not shown) because MDCK cells express a large amount of endogenous β -catenin, most of which is complexed with E-cadherin at the cell membrane. However, it is the cytoplasmic pool that is involved in β -catenin signaling and an increase in this pool was evident in both WT and S37A expressing cells as compared to the CON cell line (Fig. 1A). In addition, overnight treatment of the cells with the

1 histone deacetylase inhibitor sodium butyrate resulted in an increase in the expression
2 of the WT and S37A transgenes. Expression of the WT was enhanced to the extent that it
3 could be detected with the anti-HA antibody by immunoblotting and the S37A was
4 markedly elevated (Fig. 1B). The increased levels of the S37A protein is presumably a
5 result of its increased stability. In untreated cells a similar pattern was seen by
6 immunofluorescence. Using an antibody specific for the HA tag, only the S37A β -
7 catenin was detectable (Fig. 1 C, E, and G). A β -catenin specific antibody revealed an
8 essentially normal staining pattern in all three cell lines (Fig 1 D, F, and H). In the
9 absence of detectable nuclear staining, we wanted to confirm that β -catenin was being
10 functionally over-expressed in both the WT and S37A cell lines. To do this, LEF-
11 dependent nuclear signaling was measured using the TOPFLASH reporter construct (van
12 de Wetering et al., 1997). This reporter consists of four consensus LEF binding sites
13 placed upstream of the cFos minimal promoter. Even though the HA tag was not
14 detected in the untreated WT cell line, LEF signaling is elevated well above the control
15 (CON) (Fig. 1I) and almost to the same extent as the S37A β -catenin.

17 *β -catenin over-expression alters cell morphology*

18 Over-expression of β -catenin in MDCK cells was previously shown to alter cell
19 morphology. The pooled stable cell lines utilized in this report have essentially the same
20 morphology as the MDCKs expressing an inducible form of N-terminally truncated β -
21 catenin (Barth et al., 1997). The WT and S37A cell lines are less efficient at forming
22 tight colonies of cells as compared with CON cells (Fig. 2). In addition, the cells along

1 the edges of the WT and S37A colonies tend to extend projections more readily giving
2 them a more mesenchymal morphology.

3 The morphology of these cell lines also varied at high density. In contrast to their
4 appearance at lower density, the WT and S37A cells appeared to be more tightly adherent
5 to each other (data not shown). This is supported by the fact that these cells are
6 significantly slower to round up when trypsinized during normal cell line passaging.
7 These observations indicate that β -catenin over-expression has opposite effects on cell-
8 cell adhesion at low and high cell density. At low density, intercellular interactions are
9 reduced by β -catenin, while at high density they are strengthened.

11 *β -catenin alters cell cycle progression*

12 To characterize the distribution of these cells in the cell cycle, DNA/flow
13 cytometry analysis was performed on these cells during exponential growth phase. Both
14 of the β -catenin over-expressing cell lines had a reduced proportion of G_0/G_1 cells and an
15 increased proportion of S and G_2 cells as compared to the control cells (Fig. 3 A and
16 Table I). This suggests that, either a greater proportion of the WT and S37A cells are
17 cycling, or that the G_1 phase of the cycle is shorter in duration than it is in the control
18 cells.

19 Surprisingly, growth curves on plastic failed to demonstrate a significant
20 difference in the rate of cell accumulation in β -catenin over-expressors (Fig. 3 B). In
21 every replication of this experiment, the number of cells in the WT and S37A wells was
22 elevated (up to 50%) above the CON cells on the first day of the growth curve and

1 showed a slightly higher rate of growth for the next day or two. At this point the slopes of
2 the growth curves become parallel.

3 To determine if a difference in plating efficiency might explain the discrepancy in
4 the cell number on the first day of the growth curves, 100 cells were plated per 100 mm
5 tissue culture dish in three dishes for each cell line. The colony count provides a rough
6 estimate of the plating efficiency of the cells. This experiment revealed a small (but not
7 statistically significant) difference in plating efficiency that might account for the
8 consistent differences in the 24 hour time point (Fig. 3 C). More dramatic was an
9 obvious increase in the rate of colony growth in the β -catenin over-expressing cells. The
10 colonies from the WT and S37A cells were many fold larger than those from the CON
11 cells. The morphology of these clones provides one explanation for the difference in
12 colony size (Fig. 3 D, E, and F). While the CON cells formed tightly adhesive,
13 epithelioid colonies (Fig. 3 G), the WT and S37A cells formed a large number of colonies
14 containing a more scattered, mesenchymal phenotype (Fig. 3 H). The reduced
15 adhesiveness and apparent hypermotility may lead to this dramatic increase in colony size
16 by avoiding the contact inhibitory effect of tight cell-cell adhesion. In addition, other
17 data suggest that the WT and S37A cells have an increased proliferative rate even in the
18 presence of strong intercellular adhesion (see below).

19 *Effect of β -catenin on the expression of E-cadherin and p27*

20 Two of the phenotypic alterations caused by β -catenin over-expression involve
21 cell-cell adhesion and proliferation at different cell densities (Fig. 2 and 3). To
22 investigate the molecular mechanism by which these effects are mediated, the expression

1 of the intercellular adhesion molecule E-cadherin and the cyclin-dependent kinase
2 inhibitor p27 were determined at low and high cell density. Although one study shows
3 that E-cadherin expression is inhibited by β -catenin signaling, we found no difference in
4 protein levels at low cell density where the β -catenin over-expressing cells exhibited
5 reduced cell-cell adhesion (Fig. 9). At high density, E-cadherin protein levels in the WT
6 and S37A cells actually increased, possibly explaining the increased cell-cell adhesion in
7 that situation.

8 The inverse relationship between cell cycle variations in β -catenin and p27
9 suggested that their regulation might be coordinately regulated and may indicate the
10 mechanism by which β -catenin regulates cell cycle progression. In addition, p27
11 expression can be modulated by an E-cadherin-associated signaling pathway (St.Croix et
12 al., 1998). Since cadherins can down-regulate β -catenin signaling in some experimental
13 systems, we hypothesized that β -catenin over-expression may down-regulate p27.
14 However, p27 expression was similar in all three cell lines in both low and high density
15 conditions (Fig. 9).

16 17 18 *β -catenin promotes proliferation at high cell density*

19 The reduction in proliferative rate that non-transformed cells experience at high
20 cell density has been termed contact inhibition of growth. Although this is a widely
21 recognized phenomenon, the signaling mechanism remains unknown. To address this,
22 the MDCK cell lines were grown to confluence and cell cycle parameters were

1 monitored. Pilot experiments revealed that the WT and S37A cells shed more cells into
2 the medium than did the CON cells. To quantify this effect, cells that were two to three
3 days post-confluent were washed two times with PBS and new medium was added. The
4 medium was collected from the wells on the next day and the suspended cells were
5 counted. The number of shed cells was markedly elevated in the WT and S37A cells as
6 compared to the CON cells (Fig. 4 A). In these experiments, shedding of the S37A cell
7 line was consistently higher than in the WT cell line.

8 The hypothesis that a higher proliferative rate was responsible for the difference
9 in cell shedding was tested by performing cell cycle analysis of these cells grown three
10 days post-confluence. This analysis demonstrated that the WT and S37A cells had a
11 higher proportion of S phase and G₂ phase and a lower percentage of G₀/G₁ phase as
12 compared with the CON cells (Fig. 4 B). This cell cycle profile is precisely what would
13 be expected if the WT and S37A cells were proliferating more rapidly than the CON cells
14 and is consistent with other experiments in which the G₁/S checkpoint control regulates
15 contact inhibition (Dietrich et al., 1997; Kato et al., 1997).

16 *β-catenin attenuates the radiation-induced G₁/S cell cycle block*

17 One important aspect of cell cycle regulation is cell cycle blockade after DNA
18 damage. These blocks which occur at the G₁/S and G₂/M transitions presumably allow
19 the cell to repair its DNA before the damage-induced errors are made permanent
20 (Weinert, 1998). We postulated that β-catenin over-expression might alter the DNA
21 damage-induced late G₁ block of the cell cycle in the MDCK cells. The three cell lines
22 were γ-irradiated with 0 Gy or 5 Gy. At eight hours post-irradiation, all of the cell lines

1 show some G₁/S and G₂/M cell cycle blockade (Fig. 5). However, while the CON had
2 very few S-phase cells (5.96 %), the WT and S37A cells retained a significant number of
3 cells in S phase (15.26% and 14.99%). 24 hours after irradiation 25.2% and 21.4% of the
4 WT and S37A cells, respectively, were in S phase compared to 0.77% of CON cells.
5 Table II contains the cell cycle parameters for all of the irradiated cells. These data
6 demonstrate that the radiation-induced G₁/S block is strongly attenuated by the over-
7 expression of β -catenin and indicates that elevated β -catenin might lead to the
8 accumulation of DNA damage and increased incidence of other mutations.

9

10 *β -catenin expression fluctuates throughout the cell cycle*

11 The previously described block of G₁/S progression by APC in normal cells
12 points to a role of endogenous β -catenin in the regulation of cell cycle progression in
13 non-transformed cells (Baeg et al., 1995). Together with our demonstration that even the
14 modest elevations of β -catenin described in this study can regulate cell cycle progression,
15 this led us to investigate its level of expression throughout the cell cycle. Preliminary
16 experiments were performed with parental MDCK cells that were partially synchronized
17 in early G₁ by serum starvation. Parallel wells of cells were collected at various time
18 points after release from G₀ by the addition of serum to make whole cell or cytoplasmic
19 lysates for analysis of β -catenin protein levels. Although total β -catenin protein did not
20 vary appreciably during the cell cycle, cytoplasmic β -catenin levels increased
21 significantly from G₁ to S phase. The increase began in late G₁ and continued through S
22 phase. These pilot experiments led us to examine this phenomenon in the A1N4 cell line

1 which is easily synchronized in early G₁ by the removal of EGF from their growth
2 medium. Like MDCK cells, cytoplasmic levels of β -catenin protein increased in late G₁
3 and continued to rise in S phase (Fig. 6 A) whereas total cell β -catenin did not vary (data
4 not shown). Densitometric scanning revealed a 23-fold increase in cytoplasmic levels
5 from early G₁/G₀ to S phase (Fig. 6 B). As a control, the blot was reprobed for cyclin
6 dependent kinase inhibitor p27 (Fig. 6 A). As expected, variations in p27 were inversely
7 related to β -catenin. To determine if this oscillation in cytoplasmic β -catenin led to
8 fluctuations in β -catenin/LEF signaling, A1N4 cells were assayed for TOPFLASH
9 activity after being synchronized in G₁ phase or G₂/M phase of the cell cycle. The level
10 of β -catenin/LEF signaling corresponded with the levels of cytoplasmic β -catenin
11 measured by Western blotting (Fig. 6 C). These data indicate that oscillations in β -
12 catenin signaling may be involved in the normal regulation of cell cycle progression.

13 14 *β -catenin promotes colony formation in soft agar*

15 The ability of cells to proliferate in the absence of attachment to a solid substrate
16 correlates well with the transformed, tumorigenic phenotype. In order to assess the
17 transforming capacity of β -catenin *in vitro*, cells were suspended in 0.3% agar and
18 allowed to grow for two weeks. The ability of the WT and S37A cells to form colonies in
19 soft agar was clearly enhanced relative to the CON cells (Fig. 7 A-C). Although the CON
20 cells do exhibit a background level of colony formation, expression of the β -catenin
21 transgenes resulted in a ten to twenty fold increase in the number of colonies and an
22 obvious increase in colony size (Fig. 7 D). Multiple experiments did not demonstrate a

1 significant difference between the WT and S37A cell lines. This is the first
2 demonstration that WT or the S37A β -catenin has transforming capacity.

3 Non-transformed epithelial cells in culture require attachment to the extracellular
4 matrix for both proliferation and survival. Colony formation in soft agar requires that
5 cells overcome this requirement and prompted us to investigate the effects of β -catenin
6 on cell cycle progression and apoptosis.

7

8 *β -catenin inhibits anoikis*

9 When non-transformed epithelial cells are deprived of attachment to an
10 extracellular matrix for an extended period of time they undergo apoptosis (Frisch and
11 Ruoslahti, 1997; Frisch and Francis, 1994). This suspension-induced apoptosis has been
12 termed anoikis. As demonstrated above, most CON cells die when suspended in soft
13 agar. However, the remaining cells did contribute to a background rate of colony
14 formation. To investigate the possibility that β -catenin increases the colony-forming
15 capacity of MDCK cells by preventing anoikis, cells were cultured on a cushion of 0.8%
16 agar in normal growth medium, collected at eight hour intervals over a 24 hour period,
17 and assayed for apoptosis. Microscopic examination of the cells after sixteen and 24 hour
18 incubations revealed that the majority of the WT and S37A cells were larger and more
19 refractile to light than the CON cells (data not shown) suggesting that the CON cells were
20 preferentially undergoing apoptosis. These preliminary results were confirmed by
21 DNA/flow cytometry and Annexin V staining (Fig. 8 A, B). Both methods showed that
22 anoikis was significantly inhibited by β -catenin overexpression.

1 The results of further analysis of the flow cytometry and AnnexinV data for the
2 percentage of hypodiploid and AnnexinV-positive cells, respectively, are compiled in
3 Table II. The DNA/flow cytometry data revealed that the percentage of hypodiploid cells
4 was markedly and consistently lower in the WT and S37A cells relative to the CON cells.
5 However, these data significantly underestimates the percentage of apoptotic cells in the
6 CON samples at the 16 hour time point as the disintegrating apoptotic cells were lost
7 from the analysis. The AnnexinV assays appeared to retain these cells and probably give
8 a more accurate estimate at 16 hours.

9 As a third independent method of measuring apoptosis, nuclear morphology of
10 cells before and after suspension was analyzed by Hoechst staining. In contrast to the
11 non-suspended cells which all had normal nuclear morphology (Fig. 8 C), most of the
12 CON cells displayed characteristically shrunken apoptotic nuclei (Fig. 8 D). In contrast,
13 the nuclei of the majority of WT and S37A cells displayed a normal morphology (Fig. 8
14 E). A fraction of the cells (approximately one quarter) were apoptotic which is consistent
15 with the AnnexinV and flow cytometry results. Interestingly, a minority of CON cells
16 were found to be associated with clumps of five or more cells. Most of these cells
17 displayed normal nuclear morphology. This was a clear demonstration that cell-cell
18 adhesion can prevent apoptosis induced by suspension and this probably caused us to
19 underestimate the percentage of apoptosis among the suspended CON cells by the
20 AnnexinV and flow cytometric methodologies.

21 These data demonstrate that β -catenin overexpression may promote soft agar
22 colony formation of MDCK cells by the promotion of cell cycle progression and

1 inhibition of anoikis.

4 Discussion

5 It is suspected that the cadherin-associated protein β -catenin promotes the process
6 of carcinogenesis. The data that support this hypothesis include the following indirect
7 observations: 1. it associates with and is down-regulated by the tumor suppressor APC, 2,
8 it transduces (at least partly) the oncogenic Wnt growth factor signal to the nucleus, and
9 3, it is mutated in a significant number of human cancers (Rubinfeld et al., 1993; Su et
10 al., 1993; Cadigan and Nusse, 1997; Ilyas et al., 1997b; Fukuchi et al., 1998; Voeller et
11 al., 1998; Miyoshi et al., 1998; Palacios and Gamallo, 1998; Ilyas et al., 1997a).
12 However, no studies provide direct evidence for the transforming potential of β -catenin.
13 In addition, no investigations have addressed the question of which cellular processes β -
14 catenin may regulate to effect cellular transformation.

15 *β -catenin transforms the epithelial MDCK cell line*

16 This report characterizes phenotypic alterations that result from β -catenin over-
17 expression in a non-transformed epithelial cell line. Effects are seen in the regulation of
18 three important cellular activities/properties: proliferation, apoptosis, and morphology. It
19 demonstrates that modest β -catenin over-expression significantly enhances the ability of
20 these cells to proliferate, especially in situations that would normally inhibit the cell cycle
21 at the G₁/S transition. Most striking is the demonstration that it promotes growth in soft
22 agar, a phenotype closely correlated with tumorigenicity. Most non-transformed cells

1 require adhesion through integrin receptors to extracellular matrix components in order to
2 transit through the G₁ phase of the cell cycle (Mehta et al., 1986; Polyak et al., 1994). In
3 addition, suspension of normal, attachment-dependent cells blocks them late in G₁ phase.

4 β-catenin over-expression also resulted in increased proliferation of cells at high
5 cell density. The mechanism by which high cell density inhibits proliferation is unknown
6 but also involves a block at G₁/S. The presence of cell-cell adhesion, the reduction of
7 cell-substrate adhesion and the depletion of growth factors have all been implicated
8 (Chen et al., 1997). β-catenin's dual activities as both a regulator of cadherin-mediated
9 cell-cell adhesion and as the transducer of a mitogenic signal implicate it in this
10 regulatory process. Both cadherin and α-catenin can inhibit β-catenin signaling in other
11 experimental systems (Sehgal et al., 1997; Fagotto et al., 1998). Together with the results
12 of the present study, these data support the hypothesis that cell-cell adhesion promotes
13 the formation of cadherin/β-catenin/α-catenin complexes and that these complexes
14 negatively regulate β-catenin signaling which discourages cell cycle progression.
15 However, the fact that proliferation is reduced at high cell density as compared to
16 sparsely plated cells even in the WT and S37A cells suggests that other mechanisms are
17 also involved (for example, cell shape (Chen et al., 1997)).

18 Although growth curves did not reveal a large difference in the rate of
19 proliferation between the cell lines, cell cycle analysis demonstrated that β-catenin over-
20 expression significantly altered the distribution of these cells in the cell cycle. The
21 distribution of the WT and S37A cells was weighted heavily toward S phase and away
22 from G₁. When considered along with the other cell cycle data, it appears that β-catenin

1 over-expression expedites the G₁/S transition.

2 β -catenin over-expression also has a notable effect on cell morphology. The
3 MDCK cell line is a non-transformed epithelial line that has very strong intercellular
4 adhesion and extends cell membrane extensions only to a limited degree. β -catenin over-
5 expression converts MDCK's into a more mesenchymal cell type (Barth et al., 1997)\$.
6 At low density, cell-cell adhesion is reduced and the cells take on a more stretched shape.
7 Also, the colonies in the plating efficiency experiment (Fig. 3 H) display the same
8 morphology which suggests an increase in cell motility. These alterations in phenotype
9 resemble an epithelial to mesenchymal transition (EMT) (Huber et al., 1996). EMT's are
10 developmentally important cellular conversions especially during gastrulation, the point
11 in development at which β -catenin knockout mouse embryos are aborted. Also, an EMT
12 has been suggested to underlie the progression from benign tumor to metastatic
13 carcinoma (Sommers et al., 1991; Birchmeier et al., 1996). Indeed, it has been previously
14 suggested that β -catenin signaling may regulate this process (Sommers et al., 1994;
15 Huber et al., 1996).

16 The absence of anoikis is another characteristic of transformed cells. The present
17 study and others have shown that MDCK cells are very dependent on attachment to the
18 extracellular matrix for survival (Frisch and Francis, 1994). When individual Hoechst-
19 stained cells were examined every control cell was apoptotic, except those associated
20 with each other in clumps. The expression of the WT and S37A β -catenin transgenes
21 severely retards this process allowing approximately 75% of the single cells to survive.
22 This is a vigorous inhibition of anoikis. Taken together, the proliferation, anoikis and

1 morphology data demonstrate that these cells were clearly transformed by β -catenin.

2 The results presented in the present study differ from those published previously
3 by Young *et al* (Young et al., 1998). They reported that over-expression of the Wnt-1
4 growth factor transformed Rat-1 fibroblasts while expression of the S37A mutant form of
5 β -catenin we described previously had no effect. Two differences between the two
6 studies may explain the conflicting results. First, the morphological effects we describe
7 may only be detectable in an epithelial cell type. Second, the studies of Young *et al* were
8 carried out without serum whereas the present ones were done with serum. It is possible
9 that Wnt-1 activates parallel signaling pathways (in addition to β -catenin signaling) that
10 may circumvent the need for serum to stimulate proliferation. β -catenin's position lower
11 in the pathway may preclude the activation of such parallel pathways and, therefore it is
12 unable to stimulate proliferation in the absence of serum.

13 *β -catenin attenuates the cell's response to γ -irradiation*

14 The cell cycle blocks that characterize the response of cells to DNA damage are
15 important for the maintenance of genomic integrity. In order to prevent the permanent
16 incorporation of mutations induced by various DNA damaging stimuli, the cell cycle can
17 pause at the G₁/S and the G₂/M transitions (Weinert, 1998). During these delays, the cell
18 assesses the damage to its DNA and either repairs the damage or destroys itself. Pre-
19 mature re-entry into the cell cycle may result in the accumulation of mutations to
20 oncogenes and tumor suppressor genes which would increase the likelihood of cellular
21 transformation and cancer. The data from this study suggest that β -catenin over-
22 expression may result in the pre-mature re-entry of cells into the cell cycle following γ -

1 irradiation-induced DNA damage and thereby promote the accumulation of oncogene
2 mutations and carcinogenesis.

3 *β -catenin over-expression inhibits anoikis*

4 An association between apoptosis and the APC/ β -catenin axis has been suggested
5 previously. Re-expression of the APC gene in a tumor cell line that lacks wt APC
6 resulted in the induction of apoptosis within 24 hours (Morin et al., 1996). Since one of
7 the functions of APC is to down-regulate β -catenin, it is possible that β -catenin itself is a
8 regulator of apoptosis. Our demonstration that β -catenin alone significantly protects cells
9 from anoikis strongly implies that it can be a potent inhibitor of apoptosis. Also, during
10 the process of apoptosis, caspase-3 can cleave β -catenin protein (Brancolini et al., 1997).
11 One purpose of this cleavage may be to destroy the anti-apoptotic β -catenin signal within
12 the cell and thereby hasten the completion of the apoptotic process. The caspase-
13 mediated cleavage of Focal Adhesion Kinase (FAK) is thought to function in this manner
14 (Wen et al., 1997).

15 It has been postulated that the induction of apoptosis by the loss of appropriate
16 extracellular matrix attachment (i.e. anoikis) is a means of protecting the organism from
17 improper cell growth (Frisch and Ruoslahti, 1997). Anoikis is prevented by integrin-
18 mediated signaling. Several enzymes have been implicated as being downstream of
19 integrins in this signal transduction pathway. These include Focal Adhesion Kinase
20 (FAK), phosphoinositide-3-kinase (PI3-K), Protein Kinase B/Akt, and Integrin-Linked
21 Kinase (ILK) (Clark and Brugge, 1995; Wu et al., 1998; Giancotti, 1997). The present
22 report suggests that β -catenin may also lie downstream of integrins. Several integrin-

1 stimulated signaling pathways might lead to the induction of β -catenin signaling. One
2 possible connection between integrins and β -catenin is the integrin-activated, anti-
3 apoptotic kinase PKB/Akt. PKB is known to inhibit the activity of Glycogen Synthase
4 Kinase 3- β , a serine kinase that functions directly to reduce β -catenin protein and
5 signaling (Siegfried et al., 1992; Cook et al., 1996; Cadigan and Nusse, 1997). It is
6 possible that the result of these two inhibitory interactions is that activation of PKB by
7 integrin signaling functions to positively activate β -catenin signaling.

8 The data presented in this report describing the effects of β -catenin over-
9 expression are similar to previous reports describing the effects of integrin-linked kinase
10 (ILK) (Novak et al., 1998; Wu et al., 1998). ILK is a 59 kDa serine kinase that was first
11 described as a β_1 -integrin-associated kinase. ILK over-expression causes cells to undergo
12 an EMT and promotes their growth in soft agar. This is associated with an increase in
13 LEF-1 protein levels. As a result of increased LEF-1, β -catenin becomes completely
14 localized to the nucleus and β -cat/LEF signaling increases significantly. In addition, loss
15 of cell attachment to the underlying ECM was shown to result in a dramatic reduction in
16 LEF protein.

17 Anoikis results from the interruption of integrin-mediated signaling (Frisch and
18 Ruoslahti, 1997). In addition to ILK, the integrin-associated non-receptor tyrosine kinase
19 FAK may also be involved in the transduction of these signals because FAK signaling
20 suppresses p53-dependent apoptosis (Ilic et al., 1998). Ilic *et al* also demonstrated that an
21 atypical Protein Kinase C isoform (PKC λ/ι) is required for this p53-dependent apoptotic
22 pathway since inhibition with both chemical PKC inhibitors and a dominant-negative

1 construct protect FAK-defective cells from apoptosis. Previously, we reported that an
2 atypical PKC isoform was involved in regulating β -catenin degradation and that
3 inhibiting its activity using the same chemical PKC inhibitors resulted in the inhibition of
4 the ubiquitination and degradation of β -catenin. In addition, treatment of cells with these
5 PKC inhibitors increases β -catenin/LEF signaling (unpublished results). Taken together
6 with the present study, these data suggest that the inhibition of PKC $\lambda/1$ or another
7 atypical PKC may increase β -catenin stability and signaling leading to the suppression of
8 p53-mediated apoptosis (Fig. 10 A).

9 ***β -catenin oscillations during the cell cycle may regulate normal cellular proliferation***

10 The c-myc promoter is also regulated by the APC/ β -catenin signaling pathway
11 (He et al., 1998). The upregulation of c-myc by β -catenin may constitute the mechanistic
12 link between β -catenin and tumor formation. c-myc is potent oncogene which regulates
13 cell cycle progression. However, c-myc over-expression cannot induce cellular
14 transformation on its own. In fact, when over-expressed alone, c-myc markedly increases
15 the susceptibility of cells to apoptosis (Thompson, 1998; Steiner et al., 1996; Desbarats et
16 al., 1996). In order to transform cells, c-myc requires an accompanying survival signal to
17 prevent cells from undergoing apoptosis. Advancement through the G₁ phase of the cell
18 cycle can result in either progression into S phase or apoptosis depending on the presence
19 or absence of certain survival signals, for example IGF-1 (Evan et al., 1995). In addition
20 to stimulating c-myc, β -catenin may transduce the requisite anti-apoptotic signal that
21 would permit cell cycle progression. The increase of cytoplasmic β -catenin protein prior
22 to S phase during the cell cycle may serve this purpose in normal cells (Fig. 6).

1 Additionally, β -catenin would protect against anoikis if over-expressed in epithelial cells.

2 Our data do not demonstrate any reproducible phenotypic difference between the
3 WT and S37A expressing cells except in the measurement of protein expression and in
4 cell chedding at confluence. It is important to note that in both the WT and S37A cell
5 lines, the level of cytoplasmic β -catenin is elevated relative to the CON cells. In addition,
6 relative to control cells, β -catenin/LEF signaling is increased and similar in both the WT
7 and S37A cells. This implies that a modest increase of cytoplasmic β -catenin can result
8 in significant changes in signaling and cellular transformation and that over-expression of
9 the WT gene alone was sufficient. This may also explain how the relatively small
10 increase in endogenous cytoplasmic β -catenin that occurs before the onset of S-phase
11 may regulate the G_1/S transition in the normal cell cycle (Fig. 10 B).

12

13 The APC/ β -catenin signaling pathway has been implicated in a large number of
14 epithelial cancers (Ilyas et al., 1997b; Mareel et al., 1997). In most cases, mutations in
15 either APC or β -catenin result in stabilization of β -catenin protein and elevated β -
16 catenin/LEF signaling. However, it is not clear what role this pathway has in normal
17 cells. In this study we demonstrate that β -catenin is a potent oncogene. All of the major
18 phenomena that characterize cellular transformation, that is, soft agar growth, altered
19 morphology, inhibition of apoptosis and stimulation of cell cycle progression, can be
20 induced by the modest over-expression of β -catenin in a non-transformed epithelial cell
21 line. This clearly indicates that β -catenin plays a direct role in the process of
22 carcinogenesis and that a major component of APC function is its down-regulation.

1 These data suggest that, as an early event in the progression of colorectal cancer,
2 activation of β -catenin signaling promotes adenoma formation by promoting proliferation
3 and survival of epithelial cells in the abnormal tissue architecture of a tumor mass. In
4 addition, it may also promote the accumulation of mutations and cancer progression by
5 attenuating the DNA damage-induced G₁ cell cycle block.

6 Acknowledgements: We would like to thank the following: Dr. Mark van de Wetering
7 for supplying us with the TOPFLASH construct; Manu Kohli for his help with the
8 irradiation experiments; and Karen Creswell and Dr. Wen-Hui Zhu of the Lombardi
9 Cancer Research Center Flow Cytometry Core Facility for their help with all of the flow
10 cytometry experiments.

11 This work was funded by grants from the U.S. Department of Defense. We also
12 acknowledge the support of Dr. Marc Lippman and the Lombardi Cancer Center SPORE
13 grant.

References

- Baeg, G., Matsumine, A., Kuroda, T., Bhattacharjee, R.N., Miyaashiro, I., Toyoshima, K., and Akiyama, T. (1995). The tumor suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. *EMBO J* 14, 5618-5625.
- Barth, A.I.M., Pollack, A.L., Altschuler, Y., Mostov, K., and Nelson, W.J. (1997). NH2-terminal deletion of beta catenin results in stable co-localization of mutant beta catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J. Cell Biol.* 136, 693-706.
- Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M.M., and Birchmeier, W. (1993). Loss of Epithelial Differentiation and Gain of Invasiveness Correlates with Tyrosine Phosphorylation of the E-Cadherin/ β -Catenin Complex in Cells Transformed with a Temperature-Sensitive v-SRC Gene. *JCB* 120, 757-766.
- Birchmeier, C., Birchmeier, W., and Brand-Saberi, B. (1996). Epithelial-mesenchymal transitions in cancer progression. *Acta Anat. (Basel)* 156, 217-226.
- Brancolini, C., Lazarevic, D., Rodriguez, J., and Schneider, C. (1997). Dismantling cell-cell contacts during apoptosis is coupled to a caspase- dependent proteolytic cleavage of

- 1 beta-catenin. *J Cell Biol* 139, 759-771.
- 2
- 3 Cadigan, K.M. and Nusse, R. (1997). Wnt signaling: a common theme in animal
- 4 development. *Genes Dev* 11, 3286-3305.
- 5
- 6 Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., and Ingber, D.E. (1997).
- 7 Geometric control of cell life and death. *Science* 276, 1425-1428.
- 8
- 9 Clark, E.A. and Brugge, J.S. (1995). Integrins and signal transduction pathways: the road
- 10 taken. *Science* 268, 233-239.
- 11
- 12 Clevers, H. and van de Wetering, M. (1997). TCF/LEF factor earn their wings. *Trends*
- 13 *Genetics* 13, 485-489.
- 14
- 15 Cook, D., Fry, M.J., Hughes, K., Sumathipala, R., Woodgett, J.R., and Dale, T.C. (1996).
- 16 Wingless inactivates glycogen synthase kinase-3 via an intracellular signaling pathway
- 17 which involves a protein kinase C. *EMBO. J.* 15, 4526-4536.
- 18
- 19 Desbarats, L., Schneider, A., Muller, D., Burgin, A., and Eilers, M. (1996). Myc: a single
- 20 gene controls both proliferation and apoptosis in mammalian cells. *Experientia* 52, 1123-
- 21 1129.
- 22
- 23 Dietrich, C., Wallenfang, K., Oesch, F., and Wieser, R. (1997). Differences in the

1 mechanisms of growth control in contact-inhibited and serum-deprived human
2 fibroblasts. *Oncogene* 15, 2743-2747.

3

4 Evan, G.I., Brown, L., Whyte, M., and Harrington, E. (1995). Apoptosis and the cell
5 cycle. *Curr. Opin. Cell Biol* 7, 825-834.

6

7 Fagotto, F., Funayama, N., Gluck, U., and Gumbiner, B.M. (1998). Binding to cadherin
8 antagonizes the signaling activity of β -catenin during axis formation in *Xenopus*. *J. Cell*
9 *Biol* 132, 1105-1114.

10

11 Frisch, S.M. and Francis, H. (1994). Disruption of epithelial cell-matrix interactions
12 induces apoptosis. *J Cell Biol* 124, 619-626.

13

14 Frisch, S.M. and Ruoslahti, E. (1997). Integrins and anoikis. *Curr. Opin. Cell Biol* 9, 701-
15 706.

16

17 Fukuchi, T., Sakamoto, M., Tsuda, H., Maruyama, K., Nozawa, S., and Hirohashi, S.
18 (1998). Beta-catenin mutation in carcinoma of the uterine endometrium. *Cancer Res.* 58,
19 3526-3528.

20

21 Giancotti, F.G. (1997). Integrin signaling: specificity and control of cell survival and cell
22 cycle progression. *Curr. Opin. Cell Biol* 9, 691-700.

1

2 Gumbiner, B. (1997). Signal transduction by β -catenin. *Curr. Opin. Cell Biol.* 7, 634-640.

3

4 He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J.,

5 Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC

6 pathway [see comments]. *Science* 281, 1509-1512.

7

8 Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S., and Takeichi, M. (1992).

9 Identification of a neural alpha-catenin as a key regulator of cadherin function and

10 multicellular organization. *Cell* 70, 293-301.

11

12 Huber, O., Bierkamp, C., and Kemler, R. (1996). Cadherins and catenins in development.

13 *Curr. Opin. Cell Biol* 8, 685-691.

14

15 Ilic, D., Almeida, E.A.C., Schlaepfer, D.D., Dazin, P., Aizawa, S., and Damsky, C.H.

16 (1998). Extracellular matrix survival signals transduced by focal adhesion kinase

17 suppress p53-mediated apoptosis [In Process Citation]. *J Cell Biol* 143, 547-560.

18

19 Ilyas, M., Tomlinson, I.P., Rowan, A., Pignatelli, M., and Bodmer, W.F. (1997a). Beta-

20 catenin mutations in cell lines established from human colorectal cancers. *Proc. Natl.*

21 *Acad. Sci. U. S. A.* 94, 10330-10334.

22

- 1 Ilyas, M., Tomlinson, I.P.M., Rowan, A., Pignatelli, M., and Bodmer, W.F. (1997b).
- 2 Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc.*
- 3 *Natl. Acad. Sci. USA* 94, 10330-10334.
- 4
- 5 Kato, A., Takahashi, H., Takahashi, Y., and Matsushime, H. (1997). Contact inhibition-
- 6 induced inactivation of the cyclin D-dependent kinase in rat fibroblast cell line, 3Y1.
- 7 *Leukemia 11 Suppl 3:361-2*, 361-362.
- 8
- 9 Kemler, R. (1993). From cadherins to catenins-cytoplasmic protein interactions and
- 10 regulation of cell adhesion. *Trends Gen.* 9, 317-321.
- 11
- 12 Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W.,
- 13 Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a β -
- 14 catenin-Tcf Complex in APC^{-/-} colon carcinoma. *Science* 275, 1784-1787.
- 15
- 16 Mareel, M., Boterberg, T., Noe, V., Van Hoorde, L., Vermeulen, S., Bruyneel, E., and
- 17 Bracke, M. (1997). E-cadherin/catenin/cytoskeleton complex: a regulator of cancer
- 18 invasion. *J Cell Physiol.* 173, 271-274.
- 19
- 20 Mehta, P.P., Bertram, J.S., and Loewenstein, W.R. (1986). Growth inhibition of
- 21 transformed cells correlates with their junctional communication with normal cells. *Cell*
- 22 44, 187-196.

1

2 Miyoshi, Y., Iwao, K., Nagasawa, Y., Aihara, T., Sasaki, Y., Imaoka, S., Murata, M.,
3 Shimano, T., and Nakamura, Y. (1998). Activation of the beta-catenin gene in primary
4 hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res.* 58, 2524-
5 2527.

6

7 Morin, P.J., Vogelstein, B., and Kinzler, K. (1996). Apoptosis and APC in colorectal
8 tumorigenesis. *Proc. Natl. Acad. Sci.* 93, 7950-7954.

9

10 Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and
11 Kinzler, K.W. (1997). Activation of β -catenin/Tcf signaling in colon cancer by mutations
12 in β -catenin or APC. *Science* 275, 1787-1790.

13

14 Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. (1995). Regulation of
15 intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-
16 suppressor protein. *Proc. Natl. Acad. Sci. U. S. A.* 92, 3046-3050.

17

18 Novak, A., Hsu, S.C., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Montesano, R.,
19 Roskelley, C., Grosschedl, R., and Dedhar, S. (1998). Cell adhesion and the integrin-
20 linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc. Natl. Acad.*
21 *Sci. U. S. A.* 95, 4374-4379.

22

- 1 Orford, K., Crockett, C., Jensen, J.P., Weissman, A.M., and Byers, S.W. (1997). Serine
2 phosphorylation-regulated ubiquitination and degradation of beta catenin. *JBC* 272,
3 24735-24738.
- 4
- 5 Ozawa, M., Baribault, H., and Kemler, R. (1989). The cytoplasmic domain of the cell
6 adhesion molecule uvomorulin associates with three independent proteins structurally
7 related in different species. *EMBO J* 8, 1711-1717.
- 8
- 9 Palacios, J. and Gamallo, C. (1998). Mutations in the beta-catenin gene (CTNNB1) in
10 endometrioid ovarian carcinomas. *Cancer Res.* 58, 1344-1347.
- 11
- 12 Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996). Wnt-1 regulates free
13 pools of catenins and stabilizes APC-catenin complexes. *Mol. Cell Biol.* 16, 2128-2134.
- 14
- 15 Peifer, M., McCrea, P.D., Green, K.J., Wieschaus, E., and Gumbiner, B. (1992). The
16 vertebrate adhesion junction proteins, beta catenin and plakoglobin and the drosophila
17 segment polarity gene armadillo form a multigene family with similar properties. *J. Cell*
18 *Biol.* 118, 681-691.
- 19
- 20 Peifer, M., Sweeton, D., Casey, M., and Wieschaus, E. (1994). wingless signal and Zeste-
21 white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo.
22 *Development* 120, 369-380.
- 23

- 1 Peifer, M. (1995). Cell adhesion and signal transduction: the Armadillo connection.
2 Trends Cell Biol. 5, 224-229.
3
4 Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M., and Koff,
5 A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and
6 contact inhibition to cell cycle arrest. Genes Dev. 8, 9-22.
7
8 Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S.H., Masiarz, F.R.,
9 Munemitsu, S., and Polakis, P. (1993). Association of the APC gene product with beta-
10 catenin. Science 262, 1731-1734.
11
12 Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S.H., Masiarz, F.R.,
13 Munemitsu, S., and Polakis, P. (1993). Association of the APC gene product with beta-
14 catenin. Science 262, 1731-1734.
15
16 Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997).
17 Stabilization of β -catenin by genetic defects in melanoma cell lines. Science 275, 1790-
18 1792.
19
20 Sehgal, R.N., Gumbiner, B.M., and Reichardt, L.F. (1997). Antagonism of cell adhesion
21 by an alpha-catenin mutant, and of the Wnt- signaling pathway by alpha-catenin in
22 Xenopus embryos. J Cell Biol 139, 1033-1046.

1

2 Siegfried, E., Chou, T., and Perrimon, N. (1992). *wingless* signaling acts through *zeste-*
3 *white 3*, the *Drosophila* homolog of *glycogen synthase kinase-3*, to regulate *engrailed*
4 and establish cell fate. *Cell* 71, 1167-1179.

5

6 Sommers, C.L., Thompson, E.W., Torri, J.A., Kemler, R., Gelmann, E.P., and Byers,
7 S.W. (1991). Cell adhesion molecule uvomorulin expression in human breast cancer cell
8 lines: Relationship to morphology and invasive capacities. *Cell Growth Diffn.* 2, 365-
9 372.

10

11 Sommers, C.L., Gelmann, E.P., Kemler, R., Cowin, P., and Byers, S.W. (1994).
12 Alterations in beta-catenin phosphorylation and plakoglobin expression in human breast
13 cancer cell lines. *Cancer Res.* 54, 3544-3552.

14

15 St.Croix, B., Sheehan, C., Rak, J.W., Florenes, V.A., Slingerland, J.M., and Kerbel, R.S.
16 (1998). E-Cadherin-dependent growth suppression is mediated by the cyclin- dependent
17 kinase inhibitor p27(KIP1). *J Cell Biol* 142, 557-571.

18

19 Stampfer, M.R. and Bartley, J.C. (1988). Human mammary epithelial cells in culture:
20 differentiation and transformation. *Cancer Treat. Res.* 40:1-24, 1-24.

21

22 Steiner, P., Rudolph, B., Muller, D., and Eilers, M. (1996). The functions of Myc in cell
23 cycle progression and apoptosis. *Prog. Cell Cycle Res.* 2:73-82, 73-82.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

Su, L., Vogelstein, B., and Kinzler, K.W. (1993). Association of the APC tumor suppressor protein with catenins. *Science* 262, 1734-1737.

Thompson, E.B. (1998). The many roles of c-Myc in apoptosis. *Annu. Rev. Physiol.* 60:575-600, 575-600.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Louriero, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo co-activates transcription driven by the product of the drosophila segment polarity gene dTCF. *Cell* 88, 789-799.

Vindelov, L.L., Christensen, I.J., and Nissen, N.I. (1983). A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 3, 323-327.

Voeller, H.J., Truica, C.I., and Gelmann, E.P. (1998). Beta-catenin mutations in human prostate cancer. *Cancer Res.* 58, 2520-2523.

Weinert, T. (1998). DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. *Cell* 94, 555-558.

Wen, L.P., Fahrni, J.A., Troie, S., Guan, J.L., Orth, K., and Rosen, G.D. (1997). Cleavage of focal adhesion kinase by caspases during apoptosis. *J Biol Chem* 272, 26056-26061.

1

2 Whitehead, I., Kirk, H., and Kay, K. (1995). Expression cloning of oncogenes by
3 retroviral transfer of cDNA libraries. *Mol. Cell Biol.* 15, 704-710.

4

5 Wu, C., Keightley, S.Y., Leung-Hagesteijn, C., Radeva, G., Coppolino, M., Goicoechea,
6 S., McDonald, J.A., and Dedhar, S. (1998). Integrin-linked protein kinase regulates
7 fibronectin matrix assembly, E-cadherin expression, and tumorigenicity. *J Biol Chem*
8 273, 528-536.

9

10 Young, C.S., Kitamura, M., Hardy, S., and Kitajewski, J. (1998). Wnt-1 induces growth,
11 cytosolic beta-catenin, and Tcf/Lef transcriptional activation in Rat-1 fibroblasts. *Mol*
12 *Cell Biol* 18, 2474-2485.

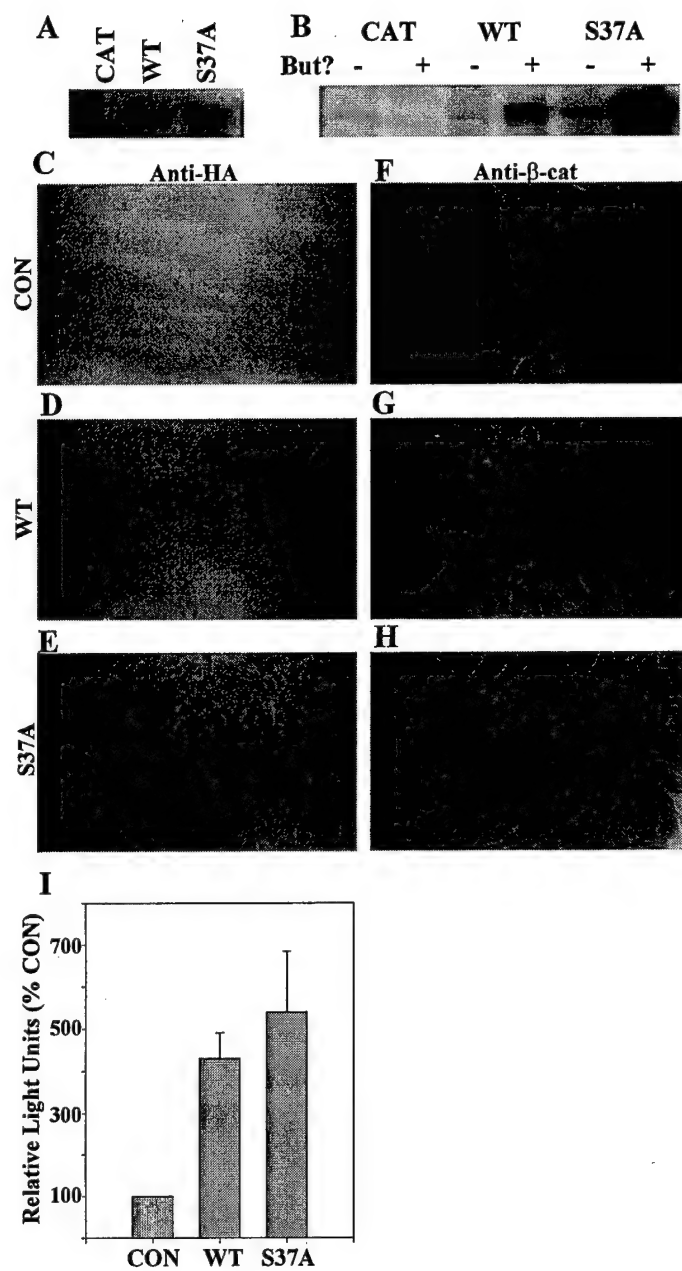
13

14

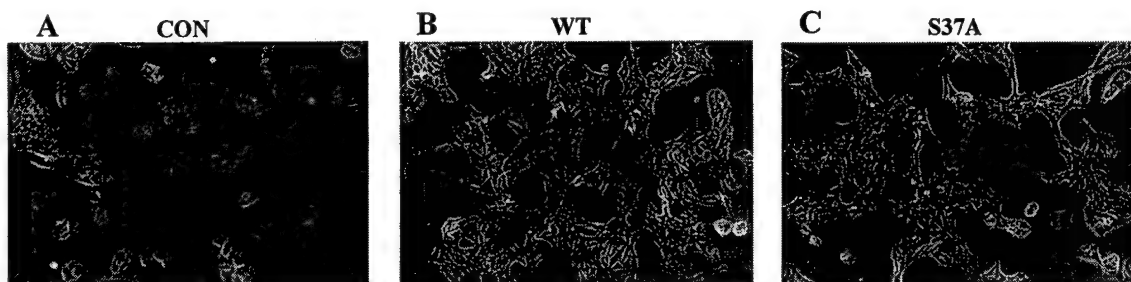
15

16

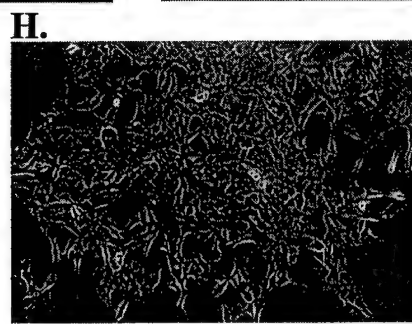
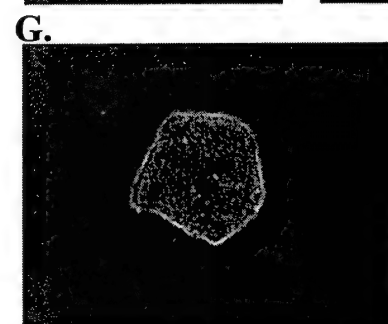
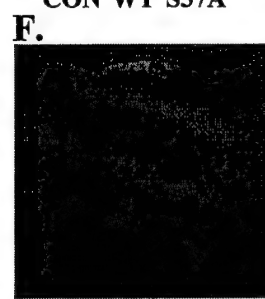
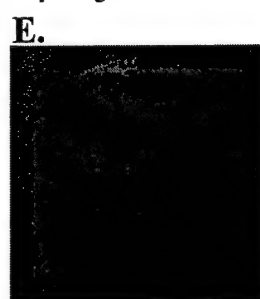
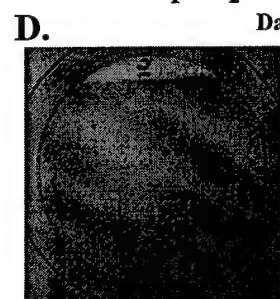
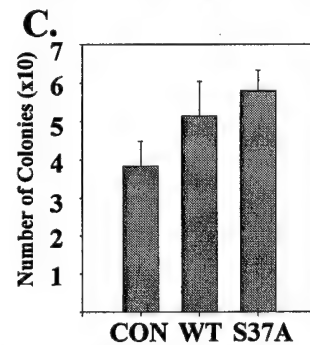
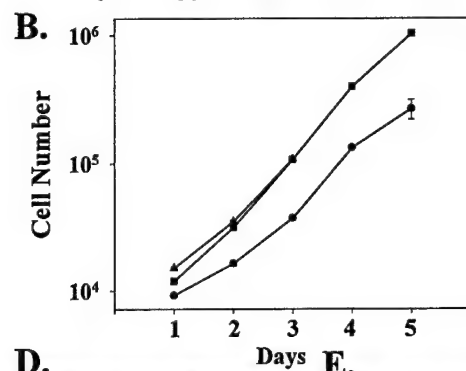
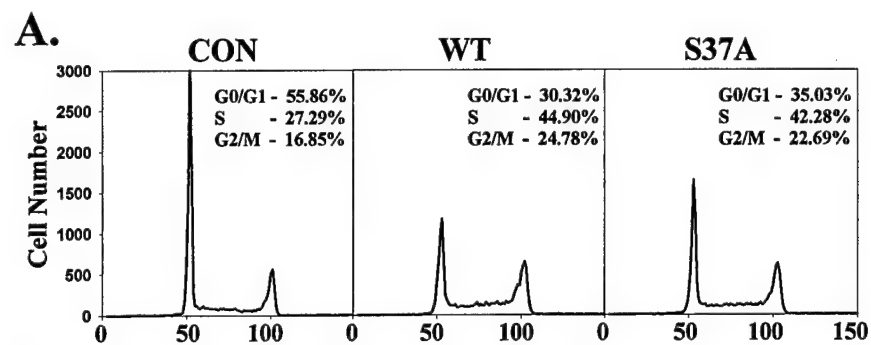
1 **Figure 1.** Expression of transgenes in MDCK pooled stable cell lines. A. Equal protein
2 from cytoplasmic extracts of CON, WT, and S37A cell lines were immunoblotted with an
3 anti- β -catenin antibody. B. Expression of HA-tagged β -catenin was determined by
4 immunoblotting equal protein from whole cell lysates of the three cell lines cultured
5 without and with sodium butyrate (But?; to enhance gene expression) with anti-HA
6 antibody (HA-11; BabCo). C-E. HA-tagged β -catenin cannot be detected in the CON (C)
7 and WT (D) cell lines by immunofluorescence. Expression is evident in the S37A (E) cell
8 line. F-H. Expression of β -catenin in the same cell lines. I. WT and S37A cell line have
9 increased LEF/ β -catenin signaling compared to CON cells.



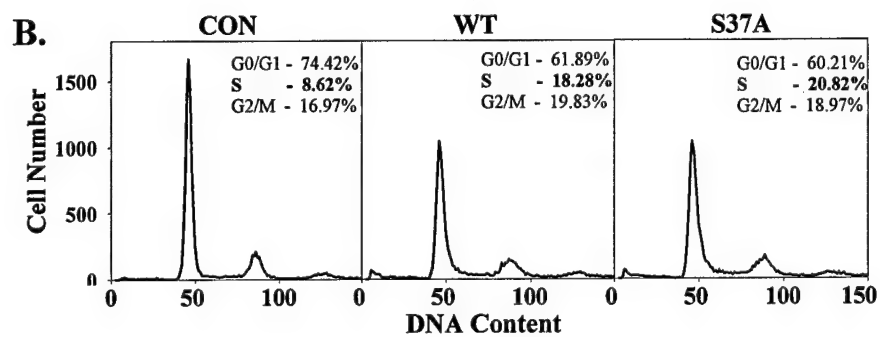
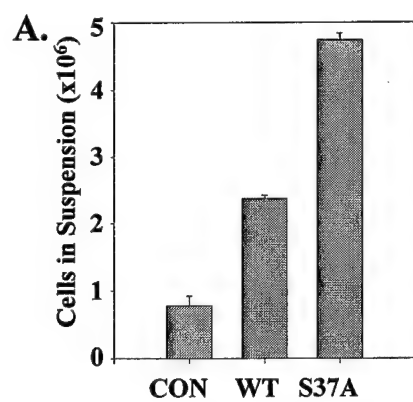
1 **Figure 2.** Exogenous β -catenin expression alters morphology of MDCK cells. Phase-
2 contrast photographs of CON (A), WT (B), and S37A (C) cell lines demonstrate the
3 effect of β -catenin over-expression on MDCK cell lines. β -catenin-expressing cells show
4 a more spindly, mesenchymal, less cell-cell adhesive morphology compared to the
5 control cells.



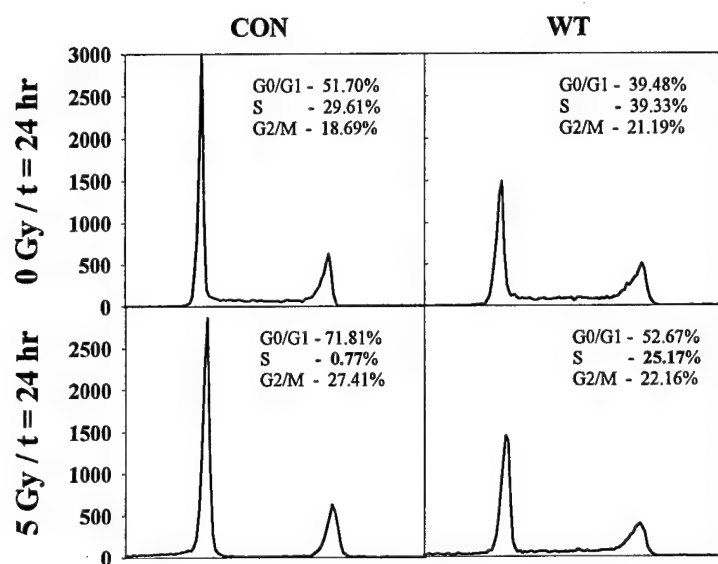
1 **Figure 3.** β -catenin over-expression alters cell cycle distribution, plating efficiency and
2 colony morphology. A. DNA/flow cytometric analysis of the three cell lines during
3 exponential growth demonstrates that the WT and S37A cell lines have a significantly
4 lower percentage of cells in the G_0/G_1 phase of the cycle and a higher percentage in both
5 S and G_2 phases of the cell cycle. B. Growth curve showing a difference in plating
6 efficiency and proliferation rate during the first two or three days. After these early time
7 points, there is little difference in growth rate. C-H. Plating efficiency assay reveals
8 changes in colony morphology. One hundred cells from each of the cell lines were plated
9 in 100-mm dishes. After eight days the colonies were stained with crystal violet, counted
10 and photographed. C. Histogram showing the colony count for the three cell lines. D-F.
11 Photographs of crystal violet stained CON (D), WT (E) and S37A (F) colonies. G, H.
12 Phase contrast photographs of representative colonies from the CON (G) and WT (H) cell
13 lines at four days. S37A colonies looked identical to the WT colony pictured



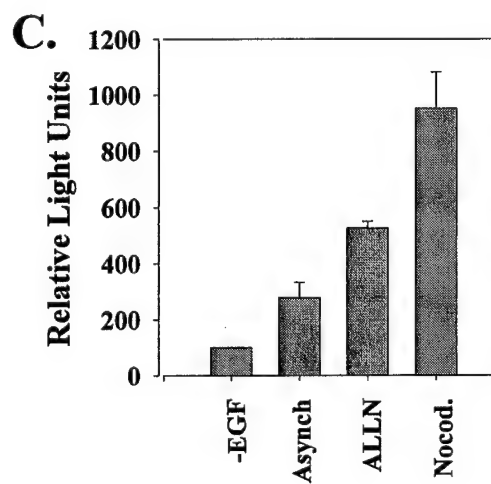
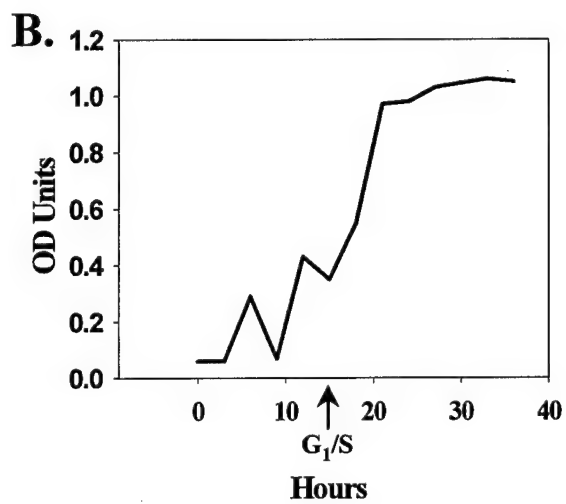
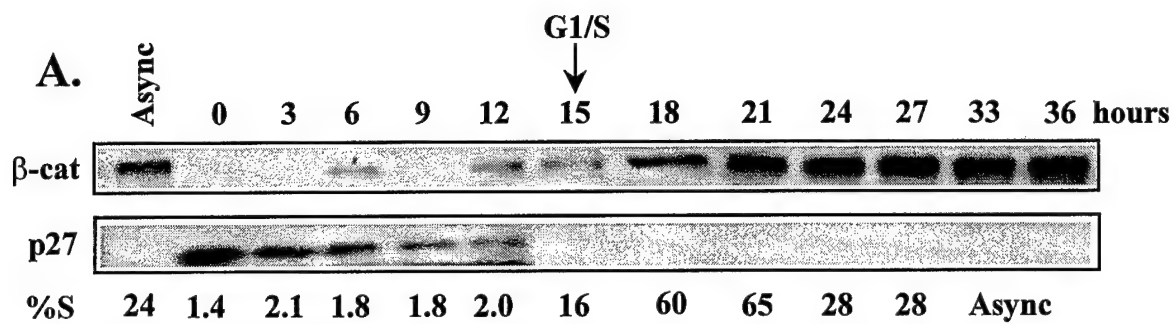
1 **Figure 4.** β -catenin over-expression promotes proliferation at high cell A. Cells were
2 cultured three days post confluence. After washing, cell shedding was measured over a
3 24 hour period by counting the number of cells suspended in the medium. B. Cell cycle
4 profiles of cells grown three days post-confluence. The S-phase percentage is increased 2
5 to 2.5-fold in WT and S37A cells relative to CON.



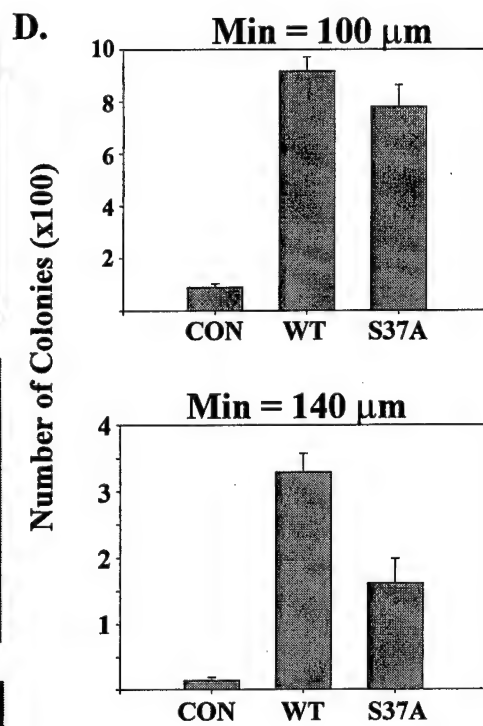
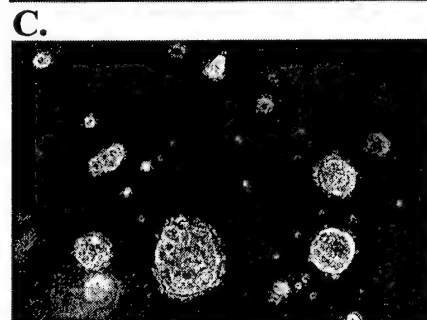
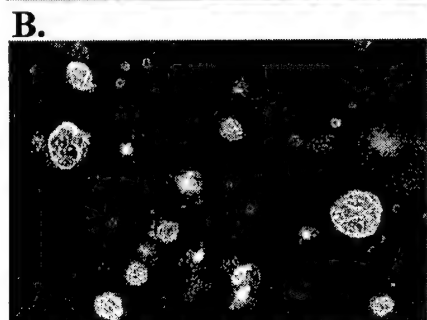
1 **Figure 5.** β -catenin over-expression attenuates the γ -irradiation G_1 cell cycle block. Cells
2 were γ -irradiated with 0 or 5 Gy. 24 hours later the cell cycle distribution was
3 determined. The unirradiated cells all had a similar profile with the characteristic
4 differences in G_0/G_1 and S phases (see Fig. 3). However, the irradiated CON sample had
5 essentially no S-phase population while the WT and S37A (not shown) samples had a
6 very significant S-phase population.



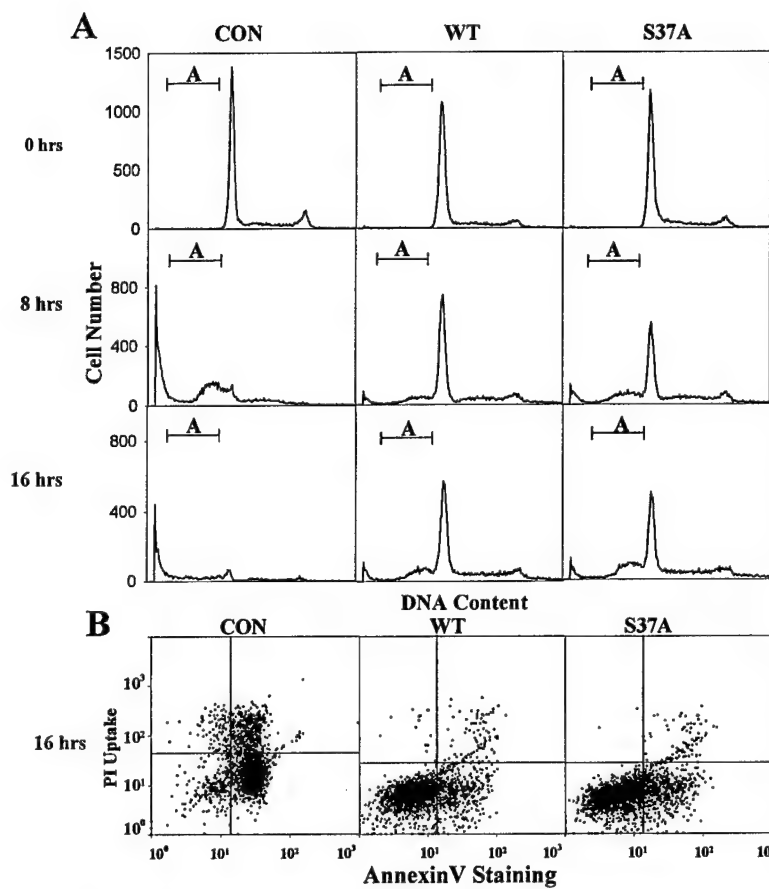
1 **Figure 6.** Cytoplasmic β -catenin oscillates during the cell cycle. A. A1N4 cells were
2 synchronized in G_0/G_1 by EGF starvation. After releasing the cells into the cell cycle by
3 the addition of EGF, cytoplasmic lysates were made every three hours and assayed for β -
4 catenin and p27 protein by immunoblotting. The distribution of cells in the cell cycle
5 was determined at each time point by analyzing parallel cell cultures by flow cytometry.
6 The percentage of S phase cells (%S) is provided. B. The level of expression was
7 determined at each time point by densitometry and the results plotted against time after
8 EGF addition. C. β -catenin/LEF signaling was measured in cells that were blocked in
9 G_0/G_1 by EGF starvation (-EGF), growing asynchronously (Asynch), blocked near the
10 S/ G_2 transition by the proteosomal inhibitor ALLN (ALLN), or blocked at G_2/M with
11 nocodazole (Nocod). The results are expressed relative to the G_0/G_1 synchronized samples.



1 **Figure 7.** β -catenin over-expression regulates soft agar colony formation. A-C. Phase-
2 contrast photographs of colonies formed by the CON (A), WT (B), and S37A (C) cell
3 lines after 14 days in soft agar. D. The number of colonies per 35-mm dish quantified by
4 the Omnicon 3600 colony counter using either 100 μ m or 140 μ m as the threshold for
5 colony diameter.



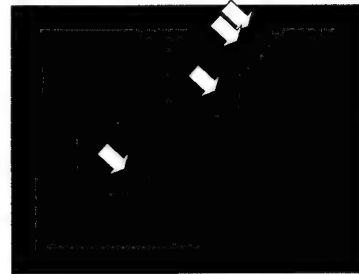
1 **Figure 8.** β -catenin expression prevents anoikis. A. DNA/ flow cytometric analysis of
2 CON, WT and S37A cells after incubation in suspension for 0, 8 or 16 hours. The
3 hypodiploid population corresponds to the apoptotic cells. The percentage in each panel
4 represents the hypodiploid fraction. B. AnnexinV and propidium iodide staining of the
5 same cells at the 16 hours also demonstrates a significant protection by β -catenin. C.
6 Hoechst staining demonstrates nuclear morphology of CON cells before suspension. All
7 nuclei look normal. WT and S37A cells looked similar. D. Hoechst staining of CON
8 cells after 16 hours in suspension. Most nuclei have a shrunken apoptotic morphology
9 (arrows). E. MUT cells after 16 hours in suspension. Most cells had the normal nuclear
10 morphology but a significant fraction (approximately one quarter) were shrunken and
11 apoptotic (arrows).



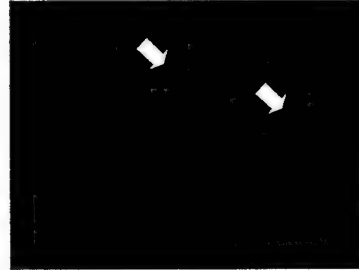
C.



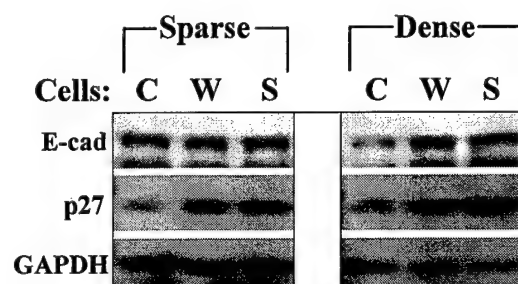
D.



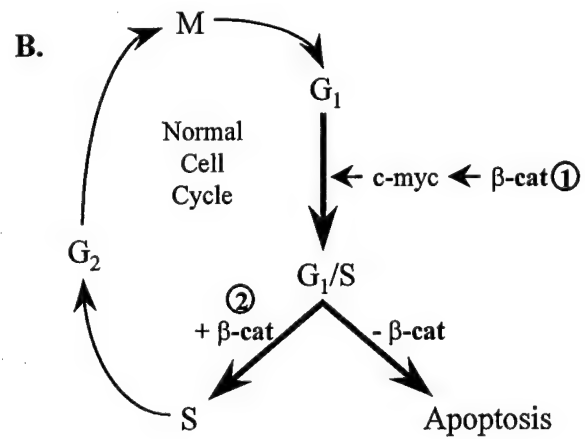
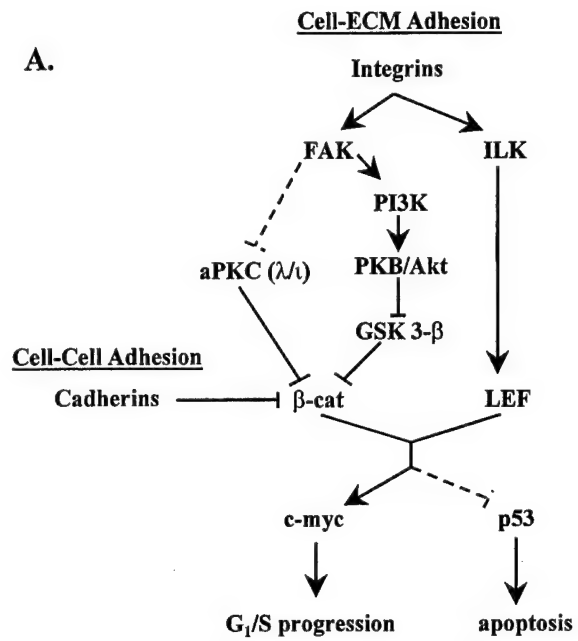
E.



- 1 **Figure 9.** The expression of E-cadherin and p27 is unchanged in the three cell lines.
- 2 Equal protein aliquots of whole cell lysates of CON, WT and MUT cells that were grown
- 3 to 75% confluency (sparse) or complete confluency (dense) were separated by SDS-
- 4 PAGE and immunoblotted for E-cadherin, p27 and GAPDH as a loading control.



1 **Figure 10.** Hypothetical signaling pathways by which β -catenin might integrate cell
2 adhesion, cell cycle and apoptosis. A. The individual regulatory events depicted by
3 unbroken arrows and blockades have been demonstrated in various published reports.
4 The broken blockades are hypothetical regulatory events suggested in the present report.
5 Integrin-activated focal adhesion kinase (FAK) activity may regulate β -catenin signaling
6 by two different pathways. In both cases, two sequential negative regulatory interactions
7 downstream of FAK may result in the activation of β -catenin signaling. By a parallel
8 pathway, integrin-linked kinase (ILK) can upregulate the expression of the transcription
9 factor LEF-1. Together, β -catenin and LEF-1 might stimulate the G_1/S transition in the
10 cell cycle (possibly via c-myc) and inhibit p53-mediated apoptosis. The inhibition of
11 apoptosis might through direct modulation of p53 action or through a parallel anti-
12 apoptotic pathway. B. β -catenin may regulate the cell cycle by two separate mechanisms.
13 1. β -catenin can stimulate the expression of c-myc which is a strong stimulator of cell
14 cycle progression. 2. The G_1/S transition represents an important decision-point for the
15 cell. It is known that this transition requires the presence of "survival factors". In their
16 absence, the cell chooses apoptosis over proliferation. β -catenin may regulate the G_1/S
17 transition as survival factor functioning to permit cell cycle progression by preventing
18 apoptosis.



1 **Table I.** Cell cycle distribution of cells with and without γ -irradiation. As compared to
2 CON cells, WT and S37A cells have an increased proportion of S-phase cells without
3 irradiation. After irradiation, the S-phase proportion of the WT and S37A cells is partly
4 reduced while in the CON cells it completely disappears. The S-phase proportions are
5 highlighted in **bold**. * These data were from a separate iteration of the same experiment.

0 Gy, 8 hr

	G₀/G₁	S	G₂/M
CON	55.86	27.29	16.85
WT	30.32	44.90	24.78
MUT	35.03	42.28	22.69
CON	48.65	5.96	45.38
WT	41.32	15.26	43.42
MUT	44.35	14.99	40.67
CON	71.81	0.77	27.41
WT	52.67	25.17	22.16
MUT	53.1*	21.37*	25.53*

5 Gy, 8 hr

5 Gy, 24 hr

1 **Table II.** β -catenin prevents anoikis as measured by DNA/flow cytometry and
2 AnnexinV labeling. The percentage of apoptotic cells in the three cell lines after different
3 periods of suspension as measured by flow cytometry (hypodiploid) or AnnexinV
4 labeling (AnnexinV positive). The percentages in **bold** demonstrate the most notable
5 effects.

% hypodiploid cells

% AnnexinV positive

	0 hrs	8 hrs	16 hrs		0 hrs	8 hrs	16 hrs
CON	3.6	56.6	44.6		1.7	50.4	74.7
WT	1.1	14.7	19.1		2.4	18.0	21.9
MUT	0.4	22.4	24.3		0.7	31.0 ⁺	24.0